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DISSERTATION

Properties of Nestin-GFP-Expressing Cells in Different Regions of Adult Murine Brain

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Abbreviations

Basic fibroblast growth factor (bFGF)
Bone morphogenic proteins (BMPs)
Brain-derived neurotrophic factor (BNDF)
5-bromo-2-desoxyuridine (BrdU)
Corpus callosum (CC)
6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX)
Central nervous system (CNS)
1,19-dioctadecyl-6, 69-di (4-sulfophenyl)-3,3,39,39-tetramethylindocarbocyanine (DiI)
Dentate gyrus (DG)
Epidermal growth factor (EGF)
Erythropoietin (EPO)
Fibroblast growth factor-2 (FGF-2)
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Granular cell layer (GCL)
Glial fibrillary acidic protein (GFAP)
Green fluorescent protein (GFP)
Insulin-like growth factor-I (IGF-I)
Intracellular Ca2+ concentration ([Ca2+] i)
Ionotropic glutamate receptors (iGluRs)
Kainate(KA)
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Metabotropic glutamate receptors (mGluRs)
Na+ voltage-gated channels (Nav)
N-methyl-D-aspartate (NMDA)
Neural stem cells (NSCs)
Neural precursor cells (NPCs)
Olfactory bulb (OB)
Oligodendroglia progenitor cells (OPCs)
Polysialylated neural cell adhesion molecule-positive (PSA-NCAM+)
Ltrans-pyrollidine-2, 4-dicarboxylic acid (PDC)
Rostral migratory stream (RMS)
Stem cell factor (SCF)
Subgranular layer (SGL)
Subgranular zone (SGZ)
Sonic hedgehog (Shh)
Stem cell factor (SCF)
Subventricular zone (SVZ)
Tetrodotoxin (TTX)
Vascular endothelial growth factor (VEGF)
Ventricular zones (VZ)
Voltage-gated Ca2+ channels (VGCCs)

Abstracts

Neurogenesis occurs throughout adulthood in two specific discrete neurogenic regions of adult mammalian brain, principally the dentate gyrus (DG) of hippocampus and the olfactory bulb (OB). Adult hippocampal neurogenesis originates from precursor cells in the dentate gyrus, and generates new granule cell neurons that become electrophysiological functional *in vivo* and were correctly integrated into the hippocampal circuitry. This process was regulated by many factors stimulation. Kempermann and colleagues propose a developmental model that takes place between these two fixed points and identify several developmental milestones based on morphology features and expression of some key markers. Originating from a presumably bipotent radial-glia-like stem cell (type-1 cell) with astrocytic properties, development progresses over at least two stages of amplifying lineage- determined progenitor cells (type-2 and type-3 cells) to early postmitotic and to mature neurons. The selection process, during which new neurons is recruited into function, and other regulatory influences differentially affect the different stages of the development (Kempermann et al., 2004).

In my PhD projects, I have concentrated on dissolving following problems:
1) What is the electrophysiology features of the presumably type1 and type2 cells in dentate gyrus?
2) Whether or not the putatively precursor cells in dentate gyrus are integrated into the brain circuitry? And how? Especially type-2 cells, since they are the first candidates to acquire neuronal properties.
3) Which types of transmitter receptors or transporters are expressed by the putatively precursor cells in the dentate gyrus?
4) What are the electrophysiology properties of nestin-GFP-expressing cells in other regions of the adult brain, including Cornu ammonis 1 (Ca1) of hippocampus, corpus callosum, amygdala, as well as striatum in physiological and ischemic condition?

Project 1:

Using a transgenic animal line in which the nestin promoter drives expression of the GFP (Yamaguchi et al., 2000), the putative precursor cells were identified. We have previously shown that nestin-GFP expressing precursor cells in the adult subgranular zone of hippocampal dentate gyrus could be divided into two distinct subpopulations based on

morphological criteria (Filippov et al., 2003) . Type-1 cells expressed glial fibrillary acidic protein, and extension of a long radial-glia-like process spanning the entire granule cell layer. They showed low proliferative activity, whereas type-2 cells had shorter horizontal or no processes, lacked expression of GFAP and were highly proliferative. The nestin-GFP-positive cell population was also distinct by its membrane current pattern: we found (1) cells with passive membrane properties similar to the classical astrocytes, (2) cells with voltage-gated channels similar to glial precursor cells or the newly described ´receptor´ astrocyte (Matthias et al., 2003), and (3) cells with sodium currents like in neurons.

Project 2:

Based on the previously results in project 1, in the following study, we employed whole cell patch clamping technique and focused on the type-2 cells since they are the first candidates to acquire neuronal properties based on our current hypothesis of neuronal development in the adult dentate gyrus (Kempermann et al., 2004). We found that radial glia-like stem cells (type1 cells) had a glial physiological phenotype; another cell population (type2 cells) was more heterogeneous including cells can generate action potentials. Some of these cells received spontaneous and evoked synaptic input, which was sensitive to GABAA, but not glutamate receptor antagonists. Both types of precursor cells expressed GABAA and glutamate receptors and glutamate transporters. Our data imply that a small, morphological distinct population of precursor cells with neuronal properties received GABAergic, but not glutamatergic input similar as in brain development.

Project 3:

An astrocyte-like cell population characterizing residual radial glia represents the neuronal progenitor cells in adult dentate gyrus. Concerning the cellular and molecular events initiating neurogenesis and controlling the transit between cellular states during maturation are poorly understood. We demonstrate that radial glia-like cells express surface-located ATP-hydrolyzing activity and are immunopositive for NTPDase2, an enzyme hydrolyzing extracellular nucleoside triphosphates such as ATP or UTP to their respective nucleoside diphosphates. NTPDase2 is expressed in the hippocampus from E17 onwards and its embryonic expression pattern mirrors dentate migration of neuroblasts. Double-immunolabeling revealed that NTPDase2 is associated with subpopulations of GFAP- and nestin-positive cells as well as by cells positive for doublecortin, which marks the

transition of progenitors to a neural phenotype. NTPDase2 is not associated with mature granule cells and S100-positive astrocytes. NTPDase2-positive cells proliferate and postmitotic cells preferentially acquire an NTPDase2-positive phenotype. We have also found the nucleotide receptors expression in the nestin-GFP-expressing cells using patch-lamp analysis of cells in hippocampal slices derived from mice transgenic for GFP under the control of the nestin promotor. Our results identify NTPDase2 as a novel marker for hippocampal progenitor cells of varying maturation state and suggest that the signaling pathway via extracellular nucleotides may play a role in the control of hippocampal neurogenesis.

Project 4:

Neurogenesis continues to occur in the adult mammalian dentate gyrus and subventricular zone. Except for the two generally accepted neurogenic regions, less is known about cell genesis in other non-neurogenesis regions of the adult brain. NG2 proteoglycan expression cells are the largest proliferative progenitor population in the postnatal CNS. In the current study, we employed the whole-cell patch-clamp approach to study nestin-GFP-expressing cells from acute slices. Data demonstrate that nestin-GFP-expressing cells in the amygdala were characteristics by the outwardly rectifying currents, functional AMPA/Kainate subtype receptor expression, and lack of glutamate transporter current. A few `neuron-like` cells elicit depolarization-induced spikes and seem receive neuronal GABAergic input. We also found the nestin-GFP-expressing cells totally express NG2. The increasing in adult-generated oligodendrocytes over time was paralleled by a decrease of BrdU+ NG2+ cells in the amygdala. Taken together, the nestin-GFP-expressing cells display OPC features and at least partly, generate oligodendrocytes in amygdala. Although, NG2 cells consistently express antigenic markers characteristic for stem or progenitor cells (Nestin, Olg2, BLBP) and neurogenic potential cells (Dcx and NeuroD), we need further studies to definite the potential property of the NG2 expressing cells in amygdala.

Project 5:

Usage of transgenic mice that express green fluorescent protein under control of the nestin promotor, we found that nestin-GFP-expressing cells present in the corpus callosum, alveus and Ca1 of hippocampus. In a combined immunohistochemical and electrophysiological approach, we also found glioblasts from the SVZ migrated through the alveus to reach CA1, where they activity-dependently generate protoplasmic astrocytes.

During the migration, these cells expressed nestin-GFP, continued to divide, and also showed astrocytic current pattern. Nestin-GFP-positive cells switched from a migratory morphology to the protoplasmic astrocytes appearance in CA1 and began to express post-mitotic astrocytic marker S100β. BrdU-labeled astrocytes were also found in CA1 after 4 weeks of BrdU injunction and intracerebroventricular dye (DiI) injections confirmed the ventricular origin of new CA1 astrocytes. Environmental enrichment regulates the astrogenesis in CA1, indicating that SVZ precursor cells contribute to physiological cellular plasticity in CA1.

Project 6:

The intermediate filament nestin is upregulated in response to cerebral ischemia, the significance of this, however, is incompletely understood. Here, we used transgenic mice that express green fluorescent protein (GFP) under control of the nestin promotor to characterize the fate of nestin-expressing cells up to 8 weeks following 30 min occlusion of the middle cerebral artery (MCAo) and reperfusion. The population of Nestin-GFP-expressing cells increased became upregulated in the ischemic lesion rim and core within 4 days, did not become TUNEL-positive and were detectable up to 8 weeks in the lesion scar. Nestin-GFP-expressing cells proliferated in situ and underwent approximately one round of cell division. They were not recruited in large numbers from the subventricular zone (SVZ) as indicated by (i) absence of co-labeling with intracerebroventricularly injected dye DiI in the majority of nestin-GFP-expressing cells and (ii) absence of migration chains. Nestin-GFP-expressing cells expressed the chondroitin sulfate proteoglycan NG2 and nestin protein, but typically lacked astrocytic markers, i.e. glial fibrillary acid protein (GFAP) or S100β. Vice versa, the majority of GFAP-expressing cells lacked nestin-expression and surrounded the ischemic lesion by 4 days. Whole cell patch clamp recordings in acute brain slices from controls demonstrated that about half of the cells had complex membrane properties and expressed AMPA receptors but lacked glutamate transporters similar to progenitor cells and a newly described subpopulation of astrocytes. By 4 days after the insult all nestin-GFP-expressing cells had acquired complex membrane properties and expressed AMPA receptors but lacked glutamate transporters (“astron”-like phenotype). We hypothesize that the change in this electrophysiological properties response induced by the ischemic insult is directed toward a network function and early neuronal differentiation of nestin-expressing cells.

1. Introduction

1.1. Endogenous Adult Neural Stem Cells

Neurogenesis, the production of new neurons, occurs only during development and stops before puberty (Rakic, 1985)(Ramóny Cajal, 1913; Jacobson, 1970) had maintained for about 100 years. The dogma had been overturned by the findings over the past decade demonstrating persistent neurogenesis and neural stem cells (NSCs) in adult brain (Gross, 2000). NSCs are the self-renewing, multipotent cells that generate neurons, astrocytes, and oligodendrocytes in the nervous system (Gage, 2000; Taupin and Gage, 2002).The first evidence for adult neurogenesis in the central nervous system (CNS) was published 40 years ago (Altman and Das, 1965; Lewis, 1968; Altman, 1969; Privat and Leblond, 1972) , but the notion that new neurons are generated in adult brain was widely accepted, at least in two discrete regions: the subgranular zone (SGZ) of the dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricles during the last decade, by using proliferation markers such as tritiatedthymidine, 5-bromo-2-desoxyuridine (BrdU) and retroviral labeling neuronal renewal.

1.1.1. Adult neurogenesis in hippocampus

Adult neurogenesis in rodents' hippocampal DG has been widely studied (Altman and Das, 1965; Kaplan and Hinds, 1977; Bayer, 1982; Kaplan and Bell, 1983, 1984; Cameron et al., 1993; Seki and Arai, 1993; Kuhn et al., 1996; Kempermann et al., 1997b). Gage and colleagues found that stem cells of SGZ cells in rodent retain the potential for self-renewal and can differentiate into neurons, astrocytes, and oligodendrocytes (Palmer et al., 1997). Stem cells originating from the SGZ of the DG migrate into the granular cell layer (GCL), where they differentiate into granule neurons(Cameron et al., 1993). Retrograde tracing studies have shown that the newly generated neuronal cells extend axons into the CA3 region of the hippocampus (Stanfield and Trice, 1988; Markakis and Gage, 1999) as soon as 4–10 days after mitosis (Hastings and Gould, 1999). In vitro, newborn neurons receive synaptic input (Stanfield and Trice, 1988; Markakis and Gage, 1999) and project functional connections(Song et al., 2002). In vivo, newborn, functional neurons are generated in SGZ integrate correctly into the hippocampal circuitry (van Praag et al., 2002).

Neurogenesis also occurs in the SGZ of tree shrews (Gould et al., 1997), nonhuman primates such as macaques (Gould et al., 1999b; Kornack and Rakic, 1999; Gould et al.,

2001), marmosets (Gould et al., 1998), and humans (Eriksson et al., 1998). Furthermore, in Old World monkeys, an in vivo study reported the generation of astrocytes and oligodendrocytes in the DG (Kornack and Rakic, 1999). SGZ cells of the adult human brain were also studied in vitro, confirming their stem cell features (Kornack and Rakic, 1999; Roy et al., 2000).

Concerning the phenotype of the primordial stem cells of the SGZ, Alvarez Buylla and colleagues demonstrated that the subgranular layer (SGL) of the dentate gyrus, which express glial fibrillary acidic protein (GFAP) and have the characteristics of astrocytes divide and give rise to new neurons (Seri et al., 2001) . They also demonstrated that a cell organization similar to that described for the SVZ exists in the hippocampus. In the SGZ, a weakly proliferative stem cell expressing GFAP (type B cell) generate to a small, dark, transient cell (type D cell), specific to the SGZ, which finally generates a GCL neuron. Usage of transgenic mice expressing green fluorescent protein (GFP) under the promoter for nestin, an intermediate filament present in progenitor cells, two reports established astrocytic features in nestin-GFP-expressing cells of hippocampal DG (Filippov et al., 2003; Fukuda et al., 2003). Kempermann and colleagues revealed the proof that newly generated neurons in the hippocampus, persisted as long as 11 months after BrdU labeling (Kempermann et al., 2003). On the contrary, other reports have exposed the new neurons transient existence in both rodents (Cameron et al., 1993; Gould et al., 1999a) and Old World monkeys (Gould et al., 1999b; Gould et al., 2001).

Adult hippocampal neurogenesis originates from precursor cells in the adult DG, generates new granule cell neurons, integrates into hippocampal circuitry and can function in cellular, network and system levels (Kempermann et al., 2004). This process can be regulated by many factors stimulation (See1.2. Regulation of adult neurogenesis). Kempermann and colleagues propose a model of the development that takes place between the origination of new generated neuron and its functional integration into brain circuitry. They identify several developmental milestones based on morphology features and expression of some key markers (Kempermann et al., 2004). Originates from a presumably bipotent radial-glia-like stem cell (type-1 cell) with astrocytic properties, development progresses over at least two stages of amplifying lineage- determined progenitor cells (type-2 and type-3 cells) to early postmitotic and to mature neurons. The selection process, during which new neurons is recruited into function, and other regulatory influences differentially affect the different stages of development. The model of six developmental milestones, which was proposed by Kempermann and his colleagues, was illustrated in

Fig.1.1 and 1.2. (STAGE 1): the division of a stem cell (type-1 cell). The type-1 cells are the radial-glia-like stem cells of the adult dentate gyrus, that giving rise to (STAGES 2–4) three consecutive stages (type-2 and type-3 cells) of putative transiently amplifying progenitor cells in the neuronal lineage, which differ by their proliferative potential and their increasing neuronal differentiation. These stages lead finally to the exit from the cell cycle and to (STAGE 5) a transient postmitotic stage express calretinin, during which network connections are established and the selection for long-term survival occurs, and finally to (STAGE 6) the stage of the terminally differentiated new granule cells express calbindin.

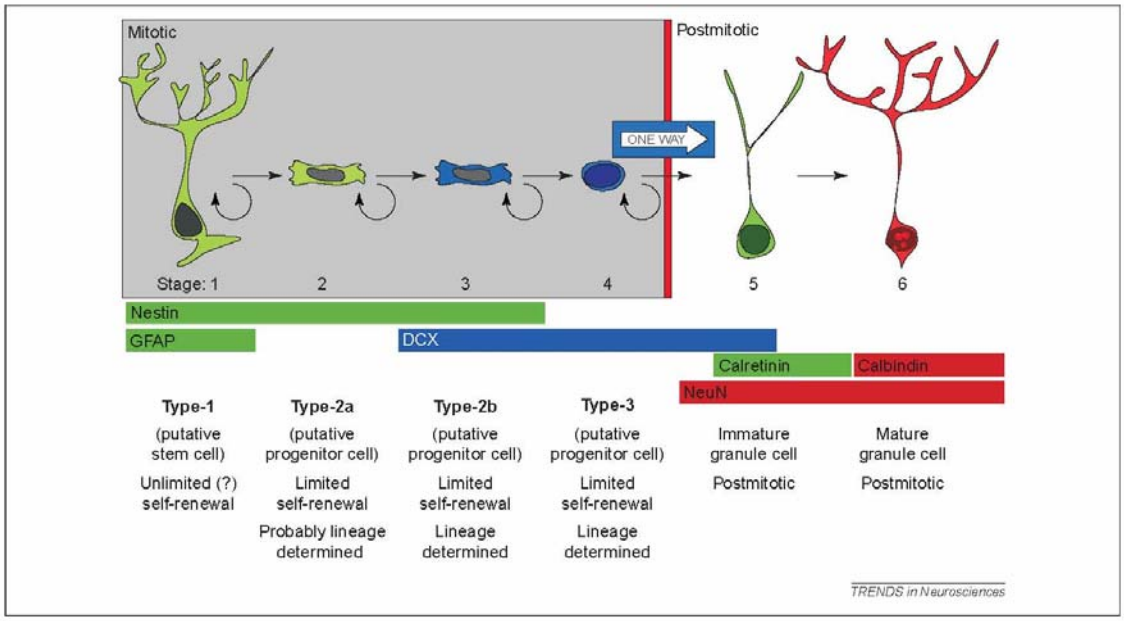

Fig.1.1. Proposed sequence of cell types in adult hippocampal neurogenesis. Six stages of neuronal development in the adult hippocampus can be readily identified on the basis of morphology, proliferative ability, and expression of markers such as nestin, glial fibrillary acidic protein (GFAP), doublecortin (DCX), calretinin, calbindin and NeuN. Development originates from the putative stem cell (type-1 cell; stage 1) that has radial glia and astrocytic properties and is probably identical to the astrocyte-like B-cell, first identified as the stem cell of this region by Seri et al. 2001. Neuronal development then progresses over three stages of putative transiently amplifying progenitor cells (type-2a, type-2b and type-3 cells; stages 2–4), which appear to be increasingly determined to the neuronal lineage because in vivo no overlap with any glial markers has been found in these cells, to an early postmitotic stage (indicated by the 'one-way' sign). This transient early postmitotic period is characterized by calretinin expression (stage 5). Confocal images of these stages are found in Figure 2, and quantification is in Figure 3. Distinction of cells as stem cells, transiently amplifying progenitor cells and lineage-determined progenitor cells is hypothetical and remains to be proven in vivo. Modified from (Brandt et al., 2003). Fig.1.1. and figure legend cited from: Kempermann, G., Jessberger, S., Steiner, B., and Kronenberg, G. (2004). Milestones of neuronal development in the adult hippocampus. Trends Neurosci *27*, 447-452

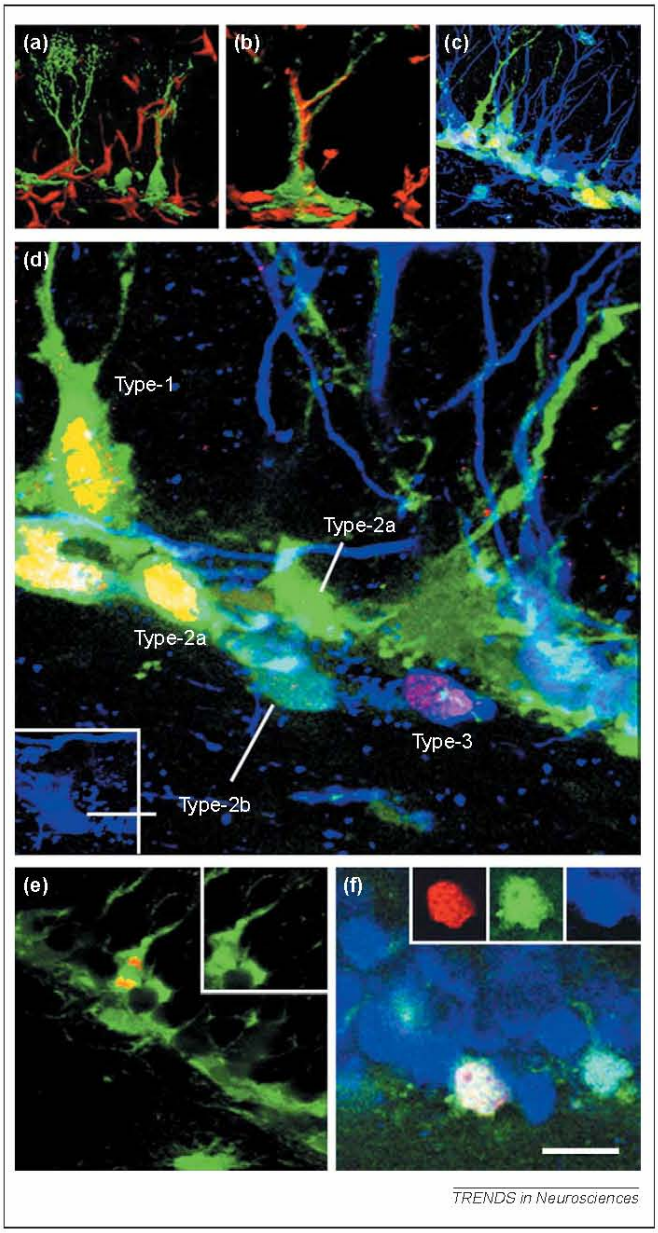

Fig.1.2. Cell types in neuronal development of the adult hippocampus. Confocal microscopic images from mouse brain depict the different cell types in adult hippocampal neurogenesis [methods are as described in (Steiner et al.2004; Kronenberg et al.2003; Brandt et al. 2003)]. (a) Type-1 cells have a radial glia-like appearance with the cell body in the subgranular zone of the dentate gyrus and a strong process reaching into the granule cell layer. They supposedly are the bipotent stem cell of this region. They express nestin [nestin green fluorescent protein (nestin-GFP; green)] and glial fibrillary acidic protein (GFAP; red). Note that not all radial-glia-like processes are nestin-positive. (b) The colocalization of GFAP and nestin within the process can be appreciated in this image. (c) Immature neurons express doublecortin (DCX; blue); this is also present in their dendrites. The young dendrites do not show a clear relationship to the similarly oriented nestin-GFP-positive processes of the type-1 cells (green). The insert shows DCX-expression (blue) in the weakly nestin-GFP-positive type-2b cell indicated. (d) The four their morphology and the absence and presence of nestin (nestin-GFP; green) and DCX (blue). For details, see Figure 1. Red identifies cells that were labeled with proliferation marker Ki67. In general, all four cell types can divide. (e) A mitotic figure in a type-1 cell suggestive of an asymmetric division, detected by immunohistochemistry for phospho-histone H3, an M-phase marker (red). The insert shows the nestin- GFP expression only. (f) The early postmitotic stage of neuronal development is characterized by expression of Ca2þ-binding protein calretinin (green) in cells that were birth-marked with the thymidine analog bromodeoxyuridine (BrdU) one week earlier (red). Blue indicates expression of the granule cell marker NeuN. Inserts display the three channels for the triple-labeled cell individually. Scale bar in (f): 100 mm (a–c), 10 mm (d), 60 mm (e) and 15 mm (f). Fig.1.2. and figure legend cited from: Kempermann, G., Jessberger, S., Steiner, B., and Kronenberg, G. (2004). Milestones of neuronal development in the adult

1.1.2. Adult Neurogenesis in SVZ

Ventricles appearances in the mammalian brain from a layer of cells surrounding fluid-filled compartments during development. This region of actively dividing and proliferating cells, the ventricular zone, generates neurons that migrate to form all structures of the brain. As the second germinal zone, SVZ, which forms later in embryogenesis beneath the ventricular zone and generates both neurons and glia. Following postnatal development, SVZ reduce until only a thin SVZ remains and persists into adulthood (Tramontin et al., 2003).

During development the SVZ generates neurons, astrocytes, and oligodendrocytes in the nervous system in fully adult mammals, but only neurogenesis persists in adulthood. In vitro studies, the adult SVZ stem/progenitor cells can be expanded in serum-free medium including epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), or the two combined and form neurospheres, which generate neurons and glia (Reynolds et al., 1992; Lois and Alvarez-Buylla, 1993; Weiss et al., 1996; Temple and Alvarez-Buylla, 1999; Gage, 2000) . In vivo studies, BrdU and retroviral tracing demonstrates that newborn neurons in the adult SVZ, migrate anteriorly into the olfactory bulb (OB), where they mature into local interneurons (Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994; Lois et al., 1996; Kornack and Rakic, 2001; Pencea et al., 2001b).

Regarding the nature of the stem cells in the SVZ, one theory contends that the NSCs of the adult SVZ are differentiated ependymal cells lining the lateral ventricle that express the intermediate filament protein nestin (Johansson et al., 1999). On the contrary, other authors state that NSCs originate from the subependymal layer of the lateral ventricle, which was regarded as astrocyte-like cells expressing GFAP and nestin in the SVZ (Morshead et al., 1994; Chiasson et al., 1999; Doetsch et al., 1999b; Laywell et al., 1999) . Alvarez-Buylla and colleagues established that the SVZ stem cell as a subependymal cell with a low proliferation rate (Doetsch et al., 1999b), with the ultrastructural characteristics of astrocytes, which extend a single cilia into the ventricle lumen through the ependymal barrier (Tramontin et al., 2003) . At least four different cell types can be defined in the SVZ–ependymal region by their morphology, ultrastructure, and molecular markers (Doetsch et al., 1997; Alvarez-Buylla and Garcia-Verdugo, 2002). The young migrating neurons (type A cells) form chains, migrate along RMS within tube-like structures formed by astrocytes (type B cells), which is the slowly proliferating stem cells expressing GFAP. The highly proliferative and rapidly dividing immature precursors (type C cells) form clusters next to the chains of migrating A cells. The SVZ is largely separated from the

ventricle cavity by a layer of ependymal cells (type E cells). B cells interact closely with E cells and occasionally contact the ventricle lumen. Whereas E cells have many long cilia, B cells contain a single short cilium (Doetsch et al., 1999a) similar to those of neural progenitors in the embryo.

The SVZ neuroblasts (type A cells) originate from the lateral wall of the lateral ventricle and cross a complex network of consistent paths before assembling the rostral migratory stream (RMS) (Doetsch and Alvarez-Buylla, 1996) and eventually lead to the olfactory bulb (OB), where they mature into local interneurons (Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994; Lois et al., 1996; Kornack and Rakic, 2001; Pencea et al., 2001b).

In the adult primate forebrain, the similar migration has also been described (Kornack and Rakic, 2001; Pencea et al., 2001a). Although a detailed study of the localization of neural stem cells in the adult human brain is still lacking, neurospheres can be generated from the adult human subependymal zone of the lateral ventricle and it has also been suggested that similar migrations may occurs in the infant human brain (Weickert et al., 2000). However, they seem to have a limited life span in culture and generate very few oligodendrocytes (Kukekov et al., 1999; Roy et al., 2000). Similarly to the case for rodents, these adult human neurospheres give rise to functional neurons and glia (Kukekov et al., 1999; Westerlund et al., 2003).

The RMS and OB also hold stem cells and considered by themselves as germinative areas(Gritti et al., 2002). Although both regions generated neurons, astrocytes, and oligodendrocytes, in vitro, the rostral part of the RMS generated more oligodendrocytes. NSCs can also be isolated from the adult human OB (Pagano et al., 2000) and present the same characteristics as human embryonic stem cells, which maintain their multipotentiality in vitro containing EGF and FGF-2 (Vescovi et al., 1999). However, embryonic stem cells keep these properties as long as 2 years, adult OB stem cells have been studied only until 40 days in vitro.

1.1.3. Adult neurogenesis in other areas of the adult brain

Adult neurogenesis also occurs in the primate cerebral cortex (Gould et al., 2001) and amygdala(Bernier et al., 2002), rodent substantia nigra (Lie et al., 2002; Zhao et al., 2003). However, other two reports strongly supported that without neurogenesis in adult primate neocortex cerebral (Kornack and Rakic, 2001; Koketsu et al., 2003); in rodent neocortex,

another study established that even after voluntary wheel running, only result in a increase in the number of newly generated cortical microglia, no any newborn neuron was observed (Ehninger and Kempermann, 2003); interestingly, with the similar methodological procedures to label proliferating cells as in (Zhao et al., 2003), the authors did not find any new dopaminergic neurons in the substantia nigra (Frielingsdorf et al., 2004).

1.2. Regulation of adult neurogenesis

Adult neurogenesis can be divided into three phases in accordance with the sequence of neurogenesis during CNS development: (a) proliferation, generation of the new cells; (b) migration toward target areas; and (c) terminal differentiation into distinct phenotypes. "Neurogenesis" means the production of neuron; it implies progression through differentiation and should not be used if only proliferation is studied. It is not yet fully understood so far, whether these phases in adult neurogenesis are regulated by the same mechanisms that regulate development, or even whether the same mechanisms regulate neurogenesis in the two adult germinal centers.

In the adult rats DG, Gould and colleagues demonstrated the evidences that many of the newborned neural precursor cells (NPCs) die between the first and second week after they are born by both [3H] thymidine-autoradiographic study (Cameron et al., 1993) and BrdU labeling method (Gould et al., 1999a) (BrdU labeled cells from 5882.8 ± 170.8 at 1 week after BrdU injection and 2130.0 ± 175.1 at two weeks after BrdU injection). Neurogenesis rate in the human dentate gyrus are similar as in adult rat brain, which the new neuronal cells that survive have been detected up to 781 days post-BrdU injection (Eriksson et al., 1998) and up to 112 days post-BrdU injection in the adult mice OB(Corotto et al., 1993). Young adult rats DG generate 9,000 new cells each day approximately, or more than 250,000 per month. This neurogenesis contributes about 3.3% of the total granule cell population per month in the young adult DG, or about 0.1% per day (Kempermann et al., 1997b; Cameron and McKay, 2001). Comparing with DG, neurogenesis rate in the olfactory bulb is higher, with approximately 80,000 new granule neurons per bulb or 1% of the olfactory granule cell population per day in young adult rodents (Kaplan et al., 1985), and nearly 0.3 % of all SVZ cells in the adult mouse may be NSCs (Rietze et al., 2001).

However, neurogenesis rate in adult brain is not stationary, but may fluctuate in response to environmental change, even slightly macroenvironmental alterations (Peterson, 2002).

Examples of positive regulators of neurogenesis include: environmental enrichment, physical activity, chronic, but not acute antidepressant treatment, caloric restriction, modulation of excitatory afferents; seizures; vitamin E deficiency; trophic factors, such as brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), insulin-like growth factor-I (IGF-I), vascular endothelial growth factor (VEGF), stem cell factor (SCF) and morphogens, such as bone morphogenic proteins (BMPs) and sonic hedgehog (Shh) signalling.

Several studies have demonstrated that an enriched environment, increase neurogenesis and, to a lesser extent, astrogliosis in the hippocampus of rodents (Kempermann et al., 1997a, b; Kempermann and Gage, 1999; Nilsson et al., 1999; van Praag et al., 2002). Physical activity also enhances neurogenesis in the DG of the hippocampus. Running can regulate hippocampal neurogenesis, synaptic plasticity, and learning (van Praag et al., 1999b; van Praag et al., 1999a). Interestingly, enriched environment and physical activity stimulate only hippocampal but not olfactory bulb neurogenesis (Brown et al., 2003a). Chronic, but not acute, antidepressant treatment, rats produced an increase in neurogenesis in a time-course consistent with the therapeutic effect (Malberg et al., 2000). Limiting food intake results in an increased survival of newly generated hippocampal neurons and also elevates hippocampal levels of brain-derived BDNF, providing evidence that this neurotrophin may act as a survival and/or differentiation factor for hippocampal neural stem cells (Lee et al., 2000) . Modulation of excitatory afferents by regulation of N-methyl-D-aspartate (NMDA) receptor activation (Cameron et al., 1995; Gould et al., 1997; Nacher et al., 2001) also altered adult neurogenesis in the dentate gyrus and may be regulated naturally by endogenous excitatory amino acids. Neural activity also promotes hippocampal neurogenesis, as shown following initiation of status epilepticus and seizure activity (Parent et al., 1997; Madsen et al., 2000; Scharfman et al., 2000; Scott et al., 2000). Adult dentate gyrus neurogenesis is enhanced in Vitamin E deficiency and vitamin E may

be an exogenous factor regulating adult neurogenesis (Ciaroni et al., 1999).

Environmental stimulis the adult neurogenesis raised another possibility that its regulation of stem cell proliferation and/or differentiation maybe under the control of expressed factors whose level of availability dictates the rate of neurogenesis(Peterson, 2002; Hallbergson et al., 2003). In addition to protecting neurons, trophic factors infusion has been shown to stimulate proliferation of adult-derived neural stem cells and to direct their differentiation toward astrocytes and oligodendrocytes and neurons. For example, in

nonpathological conditions, the application of growth factors, EGF (Craig et al., 1996; Kuhn et al., 1997) and FGF-2 (Kuhn et al., 1997) , can enhance proliferation and trigger the mobilization and differentiation of SVZ cells. The infusion of EGF increases cell proliferation in the SVZ and the olfactory tract and favors gliogenesis rather than neurogenesis (Craig et al., 1996; Kuhn et al., 1997). Whereas EGF enhances the migration and dispersion of cells in the parenchyma surrounding the olfactory tract, FGF-2 enhances cell proliferation in the SVZ and neurogenesis in the OB (Kuhn et al., 1997); (Wagner et al., 1999). Several studies have claimed that BNDF improves the recruitment of SVZ cells to the OB and stimulates their differentiation into neurons (Zigova et al., 1998; Benraiss et al., 2001; Pencea et al., 2001b). In the hippocampus, growth factors increase proliferation and neurogenesis rather than on migration. Under normal conditions, IGF-1 improved hippocampal proliferation and neurogenesis (Aberg et al., 2000; Torres-Aleman, 2000). VEGF and SCF also stimulate neurogenesis in vitro and in vivo (Jin et al., 2002a; Jin et al., 2002b).

In addition to the growth factors described above, morphogens, such as bone BMPs, Shh, regulate the proliferation and differentiation of SVZ neural stem cells. Shh, regulate neural development; also regulate the adult hippocampal neural stem cells (Lai et al., 2003) and the adult SVZ and hippocampal DG proliferating cells were enhanced by the oral administration of a Shh agonist. This effect is mediated by an up-regulation of Gli1 signalling(Machold et al., 2003). Lim et al. showed their evidence that ependymal Noggin; an antagonist of BMP, secretion creates a neurogenic niche in the SVZ by blocking BMP signaling and therefore, the BMPs and their Noggin also play a key role in the regulation of the proliferation and differentiation of adult SVZ stem/progenitor cells. SVZ cells express BMPs and their cognate receptors. BMPs potently inhibit neurogenesis both in vitro and in vivo and promote glial differentiation. In contrast, Noggin promotes neurogenesis in vitro and inhibits glial cell differentiation (Lim et al., 2000).

Examples of inhibitors of adult neurogenesis in vivo include: opiate, methamphetamine, neuroinflammation, aging, stress and serotonin depletion.

In the adult rat hippocampal granule cell layer, chronic administration of morphine decreased neurogenesis by 42% and opiate regulation of neurogenesis in the adult rat hippocampus may be one mechanism by which drug exposure influences hippocampal function (Eisch et al., 2000). In postnatal day 14 (P14) male gerbils, administration of a single non-invasive dose of methamphetamine (50 mg/kg; i.p.) interferes with adult

granule cell proliferation in the dentate gyrus (Hildebrandt et al., 1999). In the neonatal rat, postnatal day 0 (P0) and P4, there is a reduction in newly born granule cells following the recurrent seizures (McCabe et al., 2001). Palmer and colleagues demonstrated that irradiation induces neural precursor-cell dysfunction (Monje et al., 2003) and neuroinflammation alone inhibits neurogenesis and that inflammatory blockade with indomethacin, a common nonsteroidal anti-inflammatory drug, restores neurogenesis after endotoxin-induced inflammation and augments neurogenesis after cranial irradiation (Monje et al., 2003). In lipopolysaccharide-induced inflammation animal model that the researchers demonstrate that, which gives rise to microglia activation in the area where the new neurons are born, strongly impairs basal hippocampal neurogenesis in rats. The impaired neurogenesis in inflammation could also be restored by systemic administration of minocycline that inhibits microglia activation (Ekdahl et al., 2003) . Stress decreases proliferation in the SGZ in rats (Gould and Cameron, 1996; Lemaire et al., 2000), tree shrews (Gould et al., 1997; Picard-Riera et al., 2004), and marmosets (Gould et al., 1998; Picard-Riera et al., 2004), possibly through an increased concentration of glucocorticoides, but this remains to be demonstrated (Cameron and Gould, 1994). Several studies have demonstrated that aging (Kuhn et al., 1996; Kempermann et al., 1998; Cameron and McKay, 1999) and serotonin depletion (Brezun and Daszuta, 1999, 2000) inhibit the adult neurogenesis, respectively.

1.3. Expression of neurotransmitters receptors are involved in neurogenesis

In addition to the growth factors, hormones have been implied in the extrinsic regulation of cell proliferation within the developing telencephalon, the neurotransmitters, which belong to the microenvironment of neural cells *in vivo*, also play a crucial rule in regulating morphogenetic events preceding synaptogenesis such as cell proliferation, survival, migration, differentiation, and death (Cameron et al., 1998a; Nguyen et al., 2001). Neurotransmitters as a prominent candidate or transcellular signals that could influence the development of CNS cells because they surrounding neural cells throughout the CNS maturation period (Cicirata et al., 1991; Miranda-Contreras et al., 1998; Miranda-Contreras et al., 1999; Miranda-Contreras et al., 2000) and since functional ligand-gated ionic channel receptors have been described in neural progenitors before the establishment of

cortical and subcortical synapses (Sah et al., 1997; Belachew et al., 1998; Flint et al., 1998; Ma et al., 1998; Haydar et al., 2000; Ma et al., 2000; Maric et al., 2000). Furthermore, several studies strongly suggest that neurotransmitters could behave as growth regulators during specific developmental periods (LoTurco et al., 1995; Wang et al., 1996; Antonopoulos et al., 1997; Cameron et al., 1998a; Cameron et al., 1998b; Lauder et al., 1998; Ma et al., 1998; Weiss et al., 1998; Brezun and Daszuta, 1999; Butler et al., 1999; Fiszman et al., 1999; Haydar et al., 2000; Ma et al., 2000). A recently reports demonstrated that undifferentiated NPCs had a resting membrane potential of approximately -90 mV and were electrically inexcitable, application of ATP and benzoylbenzoyl-ATP evoked an inward current and membrane depolarization, whereas acetylcholine, noradrenaline, glutamate and GABA had no detectable response. After differentiation, NPC-derived neurons became electrically excitable, expressing voltage-dependent TTX-sensitive Na^+ channels, low- and high-voltage-activated Ca^{2+} channels and delayed-rectifier K^+ channels and also possessed functional glutamate, GABA, glycine and purinergic (P2X) receptors. This is a strong proof that the appearance of voltage-dependent and ligand-gated ion channels appears and the expression of neurotransmitter receptor to be an important early step in the differentiation of NPCs (Hogg et al., 2004).

1.3.1. γ-Aminobutyric acid (GABA) Receptors

GABA, the main, fast-acting inhibitory neurotransmitter in the adult neocortex (Krnjevic and Schwartz, 1967; Connors et al., 1988), is also one of the most abundant neurotransmitters detected during mammalian brain development (Cicirata et al., 1991; Miranda-Contreras et al., 1998; Miranda-Contreras et al., 1999; Miranda-Contreras et al., 2000). Three classes of GABA receptors (GABAR) are currently distinguished in the CNS: the GABAAR and GABACR, which are both ionotropic receptors, and the metabotropic GABABR (Chebib and Johnston, 1999). Among ionotropic receptors, so far six different subunit families have been identified to form CNS GABAAR receptors: α1-6, β1-3, γ1-3, δ, ε, and θ; two additional subunits, ρ1 and ρ2, have been classified as part of the GABACR (Bormann and Feigenspan, 1995; Luddens et al., 1995; McKernan and Whiting, 1996; Davies et al., 1997; Bonnert et al., 1999) .

1.3.1.1. GABA receptors expression in NSCs

During spinal and cortical neurogenesis, previously studies have depicted that the GABAAR transcripts and subunits expression in situ as components of functional GABAAR in rat neuroepithelial cells, as well as in neuroblasts and glioblasts, (LoTurco et al., 1995; Ma and Barker, 1995; Ma et al., 1998; Serafini et al., 1998b; Serafini et al., 1998a; Verkhratsky and Steinhauser, 2000). The abundance of GABA and its receptors in the early developing rodent CNS both in vivo and in vitro has led to speculations on the role of this transmitter in immature neural cell proliferation, migration, differentiation and survival.

During neural development, GABA applies a multiplicity of trophic influences through the stimulation of the GABAAR (Barker et al., 1998; Lauder et al., 1998). In vitro study revealed that the activation of functional GABAAR induces an increase in [Ca^{2+}] i in the cultures of rat embryonic telencephalic NSCs (Ma et al., 1998). In vitro, in rat embryonic hippocampal progenitor cells express functional GABAAR and the GABAergic currents remain present even after multiple passages (Sah et al., 1997). NPC-derived neurons also expressed functional GABA receptors after differentiation (Hogg et al., 2004).

1.3.1.2. GABA receptors expression in neuroblasts

Compare with mature neurons, neuroblasts have high [Cl-]i probably sustained by unrestricted, inward Cl- transport (Luhmann and Prince, 1991). Such a chloride gradient is consistent with the demonstration that either GABA or muscimol induces membrane depolarization in embryonic and neonatal cortical slices (Owens et al., 1996). In the embryonic cerebrum, Rakic and colleagues described that GABA and glutamate have opposite effects on the neural progenitor populations in VZ and SVZ. Application of either ligand to organotypic slice cultures dramatically increases proliferation in the VZ by shortening the cell cycle, whereas proliferation in the SVZ is decreased (Haydar et al., 2000). In rat embryonic neocortex (E16 and E19), an in situ study has demonstrated that during the early stages of cortical neurogenesis, both GABA and glutamate depolarize cells in the VZ and glutamate acts on AMPA/kainate receptors, while GABA acts on GABAA receptors. GABA and glutamate also increases intracellular Ca^{2+} in ventricular zone cells, through activation of voltage-gated Ca^{2+} channels. Furthermore, GABA and glutamate decrease the number of embryonic cortical cells synthesizing DNA and application of GABAA and AMPA/kainate receptor antagonists increase DNA synthesis, indicating that endogenously released amino acids is sufficient to influence neocortical progenitors in the

cell cycle (LoTurco et al., 1995). In the postnatal mouse brain neuronal progenitors in the SVZ and RMS, containing GABA and are depolarized by GABA, which may constitute the basis for a paracrine signal among neuronal progenitors (Wang et al., 2003). In the polysialylated neural cell adhesion molecule-positive (PSA-NCAM+) precursor cells from postnatal striatum, Nguyen et al. reported that GABAA receptor inhibits cell proliferation under autocrine/paracrine mechanism. They observed PSA-NCAM+ neuronal precursors from postnatal striatum expressed GABAAR subunits in vitro and in situ and that the activation of GABAAR in PSA-NCAM+ neuronal precursors inhibited cell cycle progression both in vitro and in situ (Nguyen et al., 2003).

On the contrary, GABA promotes rat immature cerebellar granule cell proliferation by indirect activation of the mitogen-activated protein kinase (MAPK) cascade (Fiszman et al., 1999). Ikeda et al. reported that the stimulation of GABAA receptors enhances survival of embryonic striatal neurons, and that the effect is mediated by Ca^{2+} influx through L-type voltage-dependent Ca^{2+} channels, initiating intracellular signaling cascades that involve activation of H-7- and genistein-sensitive protein kinases. This means that GABA not only plays the key role in progenitor cell proliferation, but also could have trophic factor-like additional functions (Ikeda et al., 1997). Behar et al. published series of evidence to demonstrate that GABA increases rat embryonic spinal and cortical neural cell motility and it's also be important for the migration and the maturation of neuronal precursors (Behar et al., 1994; Behar et al., 1996; Behar et al., 2000). In prenatal post-proliferation phase of neostriatum, GABA is also promotes survival but not proliferation of parvalbumin-immunoreactive progenitors and may also promote migration of subpopulations of interneurons that ultimately populate the ventral telencephalon (Luk and Sadikot, 2001). In cultured rat hippocampal pyramidal neurons, rat cerebellar neurons and embryonic chick tectum, GABA also modulates neuritic outgrowth(Mattson and Kater, 1989; Michler, 1990; Barbin et al., 1993). So, GABA actions may play roles in both the formation and degeneration of the neuronal circuits in which they participate in information coding.

1.3.1.3. GABA receptors expression in glioblasts

Kettenmann and colleagues found that the expression of GABAAR in rodent astrocytes in culture by electrophysiological studies (Bormann and Kettenmann, 1988). In explant and primary astrocytes cultures of rat cerebellum, hippocampus and spinal cord, using immunohistochemistry, another reports presented the expression of GABAAR (Hosli et al.,

1997). In situ studies, Butt and Jennings' reports indicated the presence of functional GABAA receptors in neonatal rat optic nerve astrocytes in situ by electrophysiological approach (Butt and Jennings, 1994). Other three reports from Kettenmann' s lab demonstrated the GABAAR expression in identified glial cells from hippocampal slices, cerebellum slice and the developing rat spinal cord slice. The pharmacological properties of the GABA response of astrocytes from the hippocampal slice is similar to that previously described for cultured astrocytes from rat cerebral hemispheres(Bormann and Kettenmann, 1988). By whole-cell recording configuration of the patch-clamp technique, they claimed that GABA receptors, which are coupled to Cl- channels, are also expressed by astrocytes in an intact tissue(MacVicar et al., 1989). The GABA-activated current was due to the activation of GABAA receptors since muscimol mimicked and bicuculline blocked the response; moreover, the reversal potential was close to the Cl- equilibrium potential (Pastor et al., 1995). GABAA receptor also present in Bergmann glial cells during development using the cerebellum slice (Muller et al., 1994).

GABAAR not only expression in rodent astrocytes in culture and in situ, but also be found in newborn rat and murine oligodendroglia progenitor cells (OPCs) in vitro (Gilbert et al., 1984; Kettenmann et al., 1984a; Belachew et al., 1998; Williamson et al., 1998) and in situ (Pastor et al., 1995). As described above in neuroblasts (LoTurco et al., 1995), Kettenmann et al. also found that GABA depolarizes rodent oligodendrocytes and astrocytes by in vitro study using cultured oligodendrocytes from mouse spinal cord (Kettenmann et al., 1984a) and differentiated, glial fibrillary acidic protein-positive astrocytes from early postnatal rat cerebral hemispheres (Kettenmann et al., 1984b). They also shown the evidence that the OPC membrane depolarization mediated by GABA seems to increase cytosolic [Ca^{2+} i] by opening of voltage-gated Ca^{2+} channels VGCCs using fura-2 fluorescence systems (Kirchhoff and Kettenmann, 1992). Mechanism of the antiproliferative actions of glutamate receptor was that the depolarization mediated by glutamate could open Nav, and increase in intracellular Na^{+} and subsequent block of outward K^{+} currents, thereby inhibiting OPC proliferation (Knutson et al., 1997). Gallo et al. indicate that glutamate regulates oligodendrogenesis specifically at the O-2A stage by modulating K^{+} channel activity (Gallo et al., 1996). Although, GABA depolarized the OPC membrane as well as glutamate, the level of depolarization induces by GABA does not reach the activation threshold of Na^{+} voltage-gated channels (Nav), at least in O-2A glial progenitor cell (Barres et al., 1990). So, for our knowledge, so far there is no any evidence about the modulation of OPC proliferation by GABA. However, GABA could promote rat immature

cerebellar granule cell proliferation by indirect activation of the mitogen-activated protein kinase (MAPK) cascade (Fiszman et al., 1999).

1.3.2. Glutamate Receptors

Glutamate is the main excitatory neurotransmitter in the mammalian nervous system (Curtis et al., 1959; Watkins, 2000). In addition to their role in neurotransmission during adulthood, increasing evidence demonstrates that glutamate also plays novel roles in morphogenesis and regulates migration, survival, differentiation, and neuritogenesis of neurons excitatory and exert neurotrophic functions in the course of CNS development (Mattson and Kater, 1987; Simon et al., 1992; Rossi and Slater, 1993; Rakic and Komuro, 1995; Behar et al., 1996; Bhave and Hoffman, 1997; Dammerman and Kriegstein, 2000). Glutamate also plays crucial modulatory role in proliferation of forebrain neuronal precursors (McDonald and Johnston, 1990; Cameron et al., 1995; LoTurco et al., 1995; Cameron et al., 1998a; Sadikot et al., 1998; Contestabile, 2000; Haydar et al., 2000; Arvidsson et al., 2001). Glutamate activates metabotropic (mGluRs) and ionotropic glutamate receptors (iGluRs). MGluRs are G protein-coupled and regulate the synthesis of various intracellular second messengers (Pin and Duvoisin, 1995) , whereas iGluRs are heteromeric ligand-gated ion channels (Dingledine et al., 1999; Le Novere and Changeux, 1999). Based on their pharmacological and electrophysiological properties, iGluRs can be subdivided into three families: AMPAR, NMDAR and KAR. These receptors are expressed not only by neurons but also by glial cells (Dingledine et al., 1999; Verkhratsky and Steinhauser, 2000).

The gating of iGluRs leads to depolarization via activation of a non-selective cationic conductance. The receptor channels are composed of four (Laube et al., 1998; Mano and Teichberg, 1998; Rosenmund et al., 1998), or five subunits (Ferrer-Montiel and Montal, 1996); (Premkumar and Auerbach, 1997), which within one family form homomeric or heteromeric receptors. Four different genes encode the AMPA subunits GluR1– GluR4 and another five genes, GluR5–GluR7, KA1 and KA2, encode kainate-preferring receptors. NMDA receptors are composed of at least six subunits: NR1, NR2A– NR2D, and NRL. The activation of NMDA receptors requires the presence of glutamate together with glycine, the latter acting as a co-agonist (Kuryatov et al., 1994; Laube et al., 1997). IGluRs are widely expressed throughout the adult and developing mammalian CNS (Bettler and Mulle, 1995; Borges and Dingledine, 1998; Ozawa et al., 1998) among which AMPARs

and KARs seem to be the first, being expressed during early mammalian CNS ontogenesis (Gallo et al., 1995; Bardoul et al., 1998a; Bardoul et al., 1998b).

1.3.2.1. Glutamate receptors expression in NSCs

Rakic and colleagues' study showed the proof that during the primate brain embryogenesis, AMPAR and KAR are especially expressed on neural precursor cells in the ventricular and subventricular proliferative zones of the developing occipital lobe of macaque monkey fetuses(Lidow and Rakic, 1995). In vitro study, using the primary cultures of embryonic rat hippocampal precursors, including NSCs, the NMDA and kainate-gated currents has been recorded by patch clamping technique (Sah et al., 1997).

1.3.2.2. Glutamate receptors expression in NPCs

Glutamate receptors and NPCs proliferation

As has discussed above, in rat E16 and E19 cortical explant neuroblasts, glutamate, actives AMPA/KAR, could inhibit the progenitor proliferation in the ventricular zone (LoTurco et al., 1995). However, glutamate have opposite effects on the two neural progenitor populations in VZ and SVZ, since glutamate agonists both increase the proliferation rate of VZ cells and decrease the SVZ cell proliferation (Haydar et al., 2000).

Neural precursor cells were isolated from the cortical neuroepithelium of embryonic day 13 rats, which were expanded in vitro in serum-free medium in response to bFGF, express functional iGluRs. AMPA/kainate and NMDA induced increases in cytosolic Ca^{2+} and inward currents were recorded only in differentiating neurons, which were double-immunostained for BrdU incorporation and neuron-specific β-tubulin (TuJ1). However, proliferating (BrdU (+) TuJ1 (-)) cells failed to respond to any ionotropic glutamate receptor agonists. Ca^{2+} imaging revealed that a subpopulation of BrdU (+) TuJ1 (+) cells also responded to AMPA, indicating the emergence of functional ionotropic AMPA/kainate receptors during terminal cell division of neuronal committed precursors (Maric et al., 2000; O'Connor et al., 2000). In the DG of hippocampus, Gould and colleagues have studied that adult neurogenesis in the dentate gyrus of the rat is regulated by afferent input, via NMDA receptors and blockade of NMDA receptors with NMDA receptor antagonists, increase in [3H] thymidine incorporation in the developing rat (Gould et al., 1994; Cameron et al., 1995; Cameron et al., 1998b). However, for the exact

mechanism by which glutamate block gyrus dentatus cell proliferation is still unknown (Cameron et al., 1998a). In the rat striatum development, glutamate promoting proliferation of striatal neuronal progenitors by an NMDA receptor-dependent mechanism, whereas AMPA-KA-mediated receptor mechanisms have no significant effect. So, the researchers concluded that glutamate plays a novel role during early development of the ventral telencephalon (Sadikot et al., 1998; Luk et al., 2003).

Glutamate receptors and NPCs survival

Balazs et al. reported that NMDA promotes the survival of cerebellar granule cells in rat cerebellar P7-8 immature granule cell culture and the influence of NMDA in promoting cell survival could be blocked by the receptor antagonist, 2-amino-5-phosphonovalerate. The effect also depended both on the concentration of NMDA and on the degree of depolarization of cells. They concluded that activation of NMDA receptor has a trophic effect on differentiating cerebellar granule cells (Balazs et al., 1988b; Balazs et al., 1988a). Blockade of the NMDA receptor by NMDA receptor antagonist CGP 39551 at developmental stages corresponding to those at which a survival dependence on the stimulation of this receptor has been demonstrated for cerebellar granule neurons explanted in culture (from postnatal day 7 to postnatal day 11 or 13), increases developmental apoptotic elimination of granule neurons(Monti and Contestabile, 2000). In the developing spinal cord neurons in vitro, low concentrations of NMDA increased neuronal survival and application of NMDAR antagonist induce neuronal cell death (Brenneman et al., 1990). In vivo studies that the blockade of NMDA receptors also increases cell death and birth in the developing rat dentate gyrus (Gould et al., 1994; Gould and Cameron, 1996).

Glutamate receptors and NPCs growth, differentiation

In vitro model, application of glutamate on postnatal rat whole-brain dissociated cell cultures 18-day-old rats promotes neuronal growth and differentiation as compared to control cultures(Aruffo et al., 1987). In hippocampal culture system, glutamate treatment by activating NMDA receptors, Induced all six immediate early genes expression, c-fos, fosB, c-jun, junB, zif/268 (also termed Egr-1; NGFI-A; Krox-24) and nur/77 (also termed NGFI-B) which playing roles in neural differentiation (Bading et al., 1995).

Activation of NMDA receptors on developing neurons may be an important mechanism for the regulation of neuronal growth and differentiation, at least in cerebellar granule cells and hippocampal pyramidal neurons. Pearce et al. studied the effect of endogenous

glutamate on neurite outgrowth from cerebellar granule cells in culture. They found the NMDA receptor antagonist APV inhibited neurite outgrowth and indicated that endogenous glutamate, possibly released by granule cells themselves, stimulated neurite outgrowth through activation of the NMDA class of glutamate receptors (Pearce et al., 1987). In hippocampal pyramidal neurons, NMDAR activation promotes the dendritic outgrowth, most likely by the intracellular pathways involved following Ca^{2+} entry through the N-methyl-D-aspartate receptor channel (Mattson et al., 1988; Brewer and Cotman, 1989; Wilson et al., 2000).

Glutamate receptors and NPCs migration

Glutamate is also play the important role in the migration of postmitotic neurons from brain germinative areas to their final destination. In cerebellar brain slices, application of NMDAR antagonists or by high Mg^{2+} concentrations decrease the rate of mice granule cell migration from the external to the internal granule layer along the process of Bergmann glia .If remove the Mg^{2+} or an increase in NMDA or glycine concentrations will increase the migration speed(Komuro and Rakic, 1993; Rossi and Slater, 1993).

1.3.2.3. Glutamate receptors expression in glioblasts

Two groups demonstrated that application of glutamate and kainate to cultured cortical astrocytes induces membrane depolarization, thus indicating that not only neurons but also astrocytes express functional iGluRs (Bowman and Kimelberg, 1984; Kettenmann et al., 1984b). Glial AMPAR activation induces an increase in $[Na^{+}]_{i}$, which in turn blocks outward rectifying K^{+} currents (Robert and Magistretti, 1997). Because of the blockage of K^{+} channels will decrease astroglial proliferation (Pappas et al., 1994), so, AMPAR activation could be involved in glial differentiation (Verkhratsky and Steinhauser, 2000). Glutamate and kainate have been shown to induce the reorganization of the astrocytic membrane and hence, under physiological conditions where glutamate is synaptically released, have been proposed to play a role in active synapse detection and stabilization (Cornell-Bell et al., 1990; von Blankenfeld and Kettenmann, 1991). Astrocytes express iGluRs and iGluRs functional properties under normal and pathology conditions were review by (Seifert and Steinhauser, 2001).

OPCs also express iGluRs, actives as AMPAR and KAR in vitro as well as in situ has been reported (Barres et al., 1990; Jensen and Chiu, 1993; Gallo et al., 1994; Patneau et al.,

1994). Kettenmann's group found that AMPA/KAR activation reversibly blocks voltage-gated K^+ outward currents(Borges et al., 1994) via Na^+ accumulation (Borges and Kettenmann, 1995) leads to speculation about AMPA/KAR function in the control of OPC proliferation and differentiation. In vitro study, Gallo's group has demonstrated that the activation of AMPA-preferring GluRs with selective agonists inhibited OPC proliferation and lineage progression through a Ca^{2+}-independent mechanism that involves Na^+ blockade of outward K^+ currents(Gould and Cameron, 1996; Knutson et al., 1997). The mechanism downstream of the blocking of outward K^+ currents seems to involve the accumulation of the cyclin-dependent kinase inhibitors p27/Kip and P21/Cip1 (Ghiani et al., 1999), known to induce a G1 arrest in the OPCs' cell cycle (Tikoo et al., 1998). In situ study, in postnatal cerebellar slices; they also reported that regulation of glutamate on OPC proliferation and differentiation (Yuan et al., 1998). Other evidences also shown that the AMPAR, KAR and/or VGCC activation contribute to a calcium-dependent regulation of c-*fos* expression and inhibit OPC proliferation (Pende et al., 1994; Liu and Almazan, 1995). In addition, Bergles et al. discovered that the existence of a rapid signalling pathway from pyramidal neurons to OPCs in the mammalian hippocampus that is mediated by excitatory, glutamatergic synapses and another report from his group, in which demonstrated that OPCs are a direct target of interneuronal collaterals and that the GABA-induced Cl(-) flux generated by these events may influence oligodendrocyte development by regulating the efficacy of glutamatergic signaling in OPCs (Bergles et al., 2000; Lin and Bergles, 2004). So, the potential roles for AMPA/KA receptors in glia include regulation of proliferation, differentiation, modulation of synaptic efficacy and the glutamate receptors on immature glia are activated through direct synaptic inputs. Therefore, glutamate and its receptors appear to be involved in a continuous crosstalk between neurons and glia during development and also in the mature brain (Gallo and Ghiani, 2000).

In contrast to the expression of AMPA/KA receptors in glial cells, the expression of NMDAR receptors is still debated. Two in vitro studies shown that NMDA activation regulate the proliferation on retinal glial cells and NMDAR activation inhibits inwardly rectifying potassium currents in human retinal Muller glial cells (Uchihori and Puro, 1993; Puro et al., 1996). Kettenmann's lab reported that distinct functional NMDA receptors expression from mouse cerebellar and neocortex slices(Muller et al., 1993; Uchihori and Puro, 1993; Schipke et al., 2001). Wang et al. demonstrate that NMDA receptor activity is necessary for the expression OPC of the highly sialylated polysialic acid-neural cell adhesion molecule (PSA-NCAM) that is required for their migration (Wang et al., 1996).

1.4. Neurogenesis following ischaemic brain insults

Ischaemic brain damage results from two types of insults. One is stroke, which was caused by a cerebral artery occlusion, leads to irreversible damage in the core region and a partially reversible injury in the surrounding penumbral zone. The second type is cardiac arrest or coronary artery occlusion, which leads to sudden and near-total cerebral blood flow interruption, causes selective vulnerable neuronal populations death, for instance hippocampal CA1 pyramidal neurons. Focal ischaemia animal models replicate the consequences of stroke, whereas global ischaemia models replicate the consequences of cardiac arrest or coronary artery occlusion (Felling and Levison, 2003; Kokaia and Lindvall, 2003). In these two reviews, authors discussed the literatures involving the effects of ischemia on the neural stem and progenitor cells in details.

1.4.1. Ischemic injury promote NPCs proliferation

Sharp's group demonstrated the increased neurogenesis following ischemia examined the hippocampus of gerbils using transient global ischemia model induced by bilateral carotid artery clamping (Liu et al., 1998). In mice, BrdU labeling cells in the DG increases from 4 to 9 days after transient global ischemia (Takagi et al., 1999). In rats, after ischemia which was induced by usage of the temporary occlusion of the two carotid arteries combined with arterial hypotension, increasing numbers of BrdU expressing cells in the hippocampus are observed as early as 3 days, with a peak at 7 days till 4 weeks (Kee et al., 2001). Middle cerebral artery occlusion (MCAo) for 90 min in the rat induced ischemia injury, enhanced BrdU incorporation into the cells, in the subgranular zone of the DG and the rostral subventricular zone (Jin et al., 2001). And the BrdU labeling cells recovered to control levels by 2 weeks after insults and also be confirmed by another report (Takasawa et al., 2002). Although, several reports have demonstrated that the increased proliferation in the ipsilateral SVZ after MCAo peaks at 7 days and will last till 14 days (Zhang et al., 2001; Li et al., 2002; Takasawa et al., 2002), another report places the peak at 14 days using the similar model (Jin et al., 2001).

NPCs structured the majority of the proliferating population, in addition to the presentation of increased proliferation in the germinal zones of the brain after ischemic injury. Immunofluorescence study in gerbils after transient global ischemia 15 days exposes no

expression of NeuN, a mature neuronal marker, and GFAP, a mature astrocytic marker, in the BrdU positive population of the hippocampus (Liu et al., 1998). Another report also showed that BrdU positive cells did not label with NeuN and Hu, another neuronal marker in either the hippocampus or the SVZ 1 week after MCAo in rats (Jin et al., 2001), this data further verified the hypotheses that progenitor cells rather than mature cells proliferate in response to ischemic injury.

Cell death is not required for the increase of the proliferating cells after ischemia injury. Proliferating cells after ischemia express immature cells markers, and application of selective NMDA receptor antagonists abrogate cell proliferation, even in either the CA1 region or the dentate granule cell layer in which no cell death occurs after MCAo (Arvidsson et al., 2002). Another study demonstrates that administration of either NMDA or AMPA/kainate glutamate receptor antagonists, which assuages hippocampal damage during transient forebrain ischemia, also decreases the cell proliferation associated with the insult (Bernabeu and Sharp, 2000). In the contralateral hemisphere in models of unilateral ischemic injury, some reports also supporting that cell death is not required for the increased proliferation (Jin et al., 2001; Zhang et al., 2001; Takasawa et al., 2002).

1.4.2. NPCs migrate from proliferative zones to damaged regions following ischemic insult

Newborned cells after MCAo, which were characteristiced by BrdU+/DCX+, migrate away from the SVZ to the striatum of the ipsilateral hemisphere in adult rats. On the contrast; DCX immunoreactivity is restricted to the SVZ in the contralateral hemisphere (Arvidsson et al., 2002). After MCAo in adult rats, PSA-NCAM, a cell adhesion molecule involved in migration was increased expression (Stoll et al., 1998; Sato et al., 2001; Zhang et al., 2001), even though PSA-NCAM is also highly expressed by reactive astrocytes that are certainly present in the ischemic brain(Stoll et al., 1998).

Nakatomi et al. demonstrated the strong evidence for the progenitors migration into the damaged hippocampus after transient global ischemia in adult rats. After 2 days of the injection of lipophilic fluorescent dye DiI into the lateral ventricle, Dil+ cells were found to be restricted to the posterior periventricular area, but by 4 weeks after ischemia, many DiI+ cells were observed, which were NeuN positive in the damaged CA1 region of the hippocampus (Nakatomi et al., 2002).

1.4.3. Differentiation and maturation of NPCs after ischemic insult

Compared with the contralateral striatum and sham-operated controls, in the ipsilateral striatum, 31-fold increase in the absolute number of BrdU+/NeuN+ cells. BrdU+/NeuN+ cells at 4 weeks was 9.7-fold higher than at 2 weeks after stroke, indicating that progenitor cells had maintained to mature over the 4-week recovery period. Compatible with the interpretation that newborn neurons were maturing within the damaged striatum, BrdU+/NeuN+ cells stained for Meis2 and Pbx, two markers that are expressed by developing striatal cells. Furthermore, at 5 weeks of recovery, 42% of the BrdU+/NeuN+ cells were also positive for DARPP-32, a marker of mature mediumsized spiny neurons characteristic of the striatum (Arvidsson et al., 2002). In adult rats, after transient global ischemia, BrdU+ cells initially express Pax6 and Emx2, markers of immature cells. By 4 weeks after the insult, some BrdU+ cells migrate to the damaged CA1 region of the hippocampus and express mature neuronal markers, including NeuN, Tuj1, and Hu (Nakatomi et al., 2002). These two reports demonstrate that some of the newborned cells after an ischemic insult differentiate into a mature neuronal phenotype in a regionally appropriate manner, both in striatum and hippocampus. The number of NG2+ OPCs surrounding the infarct was also increased within 2 weeks after MCAo (Tanaka et al., 2000), for the astrocytic response to the ischemia demage was described in the two reviews (Stoll et al., 1998; Anderson et al., 2003).

1.4.4. Regulation of adult neurogenesis after ischemic insults

Glutamatergic mechanisms are involved in SGZ neurogenesis regulation following global and focal ischaemia (Bernabeu and Sharp, 2000; Arvidsson et al., 2001). Application the antagonists of both NMDA and AMPA receptors at the time of global ischaemia prevented the increase of neurogenesis as well as the CA1 neuronal death. Arvidsson et al. found that focal ischaemia after MCAO that did not cause ischaemic damage to the hippocampus gave rise to neurogenesis, which was completely suppressed by NMDA but not AMPA receptor blockade. These findings suggest that the mechanisms modulating SGZ neurogenesis following MCAO are at least partly different from those following global ischaemia. The marked stimulation factor of neurogenesis by activation of NMDA receptors following ischaemia is mediated by the increased levels of certain growth factors, such as, FGF-2 and BDNF, which was evoked by glutamate (Arvidsson et al., 2001).

Greenberg's group found that hypoxia in cortical cultures triggered neurogenesis, which was regulated through increased FGF-2 and stem cell factor (SCF) levels and intraventricular administration of SCF gave rise to an increased number of proliferated cells co-expressing a neuronal marker in the SVZ and SGZ (Jin et al., 2002b). In FGF-2 knockout mice, the increase of neurogenesis in the SGZ following MCAO was attenuated (Yoshimura et al., 2001).

In intact, adult rats, some reports raised the possibility that BDNF could also promote neurogenesis after ischaemic brain insults in the RMS and olfactory bulb, striatum, septum, thalamus and hypothalamus by intraventricular infusion of BDNF protein or overexpression of the BDNF gene in the ventricular zone (Zigova et al., 1998; Benraiss et al., 2001; Pencea et al., 2001a). But, long-term delivery of BDNF to the hippocampus counteracts neuronal differentiation but not cell proliferation and survival of newly generated cells in the DG after global ischaemia (Larsson et al., 2002).

Shingo et al. found that Erythropoietin (EPO) is another factor that probably regulated neurogenesis after ischaemia, which is produced as part of the ischaemic-hypoxic response and intraventricular infusion of EPO and EPO antibodies leads to increased and decreased the number of olfactory bulb neurons from SVZ, respectively (Shingo et al., 2001).

Another important mediator of the NPCs response to ischemic insults is the inducible form of nitric oxide synthase (iNOS). INOS was high expressed in the ipsilateral DG and Inhibition of iNOS activity without altering its expression attenuates the level of BrdU incorporation after MCAo in adult rats. In null mutant mice lacking the iNOS gene, increased neurogenesis was not observed after focal cerebral ischemia and the infarct volume is also significantly decreased in these animals (Zhu et al., 2003).

1.5. Reactive astrocytes following the brain injury

1.5.1 Bi-directional communication between neuron and glia

Based on the morphology features and spatial organization, astrocytes are classically divided into three major types: radial astrocytes with long unbranched processes, surrounding ventricles, protoplasmic astrocytes have a bushy morphology with numerous highly branched short processes, located in gray matter and fibrous astrocytes display more stellate shape with smooth, long processes are less branched, which located in white matter

(Reihenbach and Wulburg, 2004). The dogma that astrocyte merely providing the structural support for neurons has been overturned by the increasing evidences, which has now been demonstrated that astrocytes are crucial to the normal function of the CNS (Hertz et al., 2000; Araque et al., 2001; Bezzi et al., 2001; Bezzi and Volterra, 2001; Fields and Stevens-Graham, 2002). Astrocytes transfer energy substrates to neuron (Forsyth, 1996) and also provide neurons with substrates for neurotransmitters and antioxidants (Kirchhoff et al., 2001). Astrocytes also play the key rule in the maintenance of the extracellular environment by spatially buffering K^+ and uptake glutamate. In addition to express GABA and glutamate receptor in astrocytes as discussed above, astrocytes also express numerous of other neurotransmitter receptors ion channels and second messenger systems and thereby response and integrate input from neuron (Nilsson et al., 1991; Nilsson et al., 1993; Porter and McCarthy, 1997; Verkhratsky and Steinhauser, 2000). Astrocytes have processes that enwrap synapses, regulate the formation and function synapses (Pfrieger and Barres, 1996; Araque et al., 1999b; Araque et al., 1999a; Ventura and Harris, 1999) and also can respond to axonal stimulation with glutamate receptor-dependent Ca^{2+} elevations in acutely isolated brain slices (Bezzi et al., 1998).

Shelton et al. found that astrocytes have acetylcholine and histamine receptors coupled to Ca^{2+}. Given that Ca2+ elevations in astrocytes trigger neurotransmitter release, it is possible that these astrocyte receptors modulate neuronal activity (Shelton and McCarthy, 2000).

Norepinephrine (NE) release and alpha 1-adrenoceptor stimulation in neuron-astrocyte communication in the mature CNS (Duffy and MacVicar, 1995). Kang et al. show their finding that interneuronal firing elicits a GABAB-receptor-mediated elevation of calcium in surrounding astrocytes, which in turn potentiates inhibitory transmission. Therefore they conclude that astrocytes may be a necessary intermediary in activity-dependent modulation of inhibitory synapses in the hippocampus (Kang et al., 1998). Some researches demonstrated that the elevations in astrocytes Ca^{2+} levels in the cells culture and brain slices can lead to astrocytes-to-neuron signaling through Ca^{2+}-dependent release of glutamate (Parpura et al., 1994; Muyderman et al., 2001). With the dual capacity of glutamate release system and glutamate uptake, gives astrocytes the potential possibility to locally modulate neuronal excitability and synaptic transmission (Araque et al., 1998; Haydon, 2001).

1.5.2. Reactive astrogliosis following the brain injury

Following the brain injury, surviving astrocytes in the damaged region start to exhibit hypertrophy and proliferation, called reactive astrogliosis (Ridet et al., 1997), which was not only characterized by the hypertrophy of astrocytes as well as by the proliferation of microglial cells and astrocytes. Reactive astrocytes influence neuronal survival after brain injury including glutamate uptake, glutamate release, free radical scavenging, water transport and production of cytokines and nitric oxide (Chen and Swanson, 2003). In the healing process of damaged tissue, astrocytes express surface molecules and produce various neurotrophic factors and cytokines to influence neurite outgrowth, synaptic plasticity, or neuron regeneration (Chen and Swanson, 2003); (Ridet et al., 1997) and also potentially recapitulate ontogeny following brain injury, i.e. the re-expression of developmental proteins may indicate active reconditioning that could promote cell survival (Cramer and Chopp, 2000).

2. Experimental Procedures

2.1. Animals

Nestin promoter–GFP transgenic mice were constructed by ligation of enhanced GFP cDNA (Clontech, Palo Alto, CA) with the nestin promoter region and the enhancer region of the nestin gene spanning the 3' part of the second exon to the 5' part of the third intron and the rabbit polyadenylation site (Fig.2.1) (Yamaguchi et al., 2000). The generation of mice expressing GFP under the control of nestin gene regulatory regions has been described in detail previously (Filippov et al., 2003; Fukuda et al., 2003);(Yamaguchi et al., 2000). The mice were bred at the animal facility at the MDC. All applicable federal and local regulations of animal welfare were followed. The mice lived in standard laboratory housing conditions with a light/dark-cycle of 12 h each and food and water.

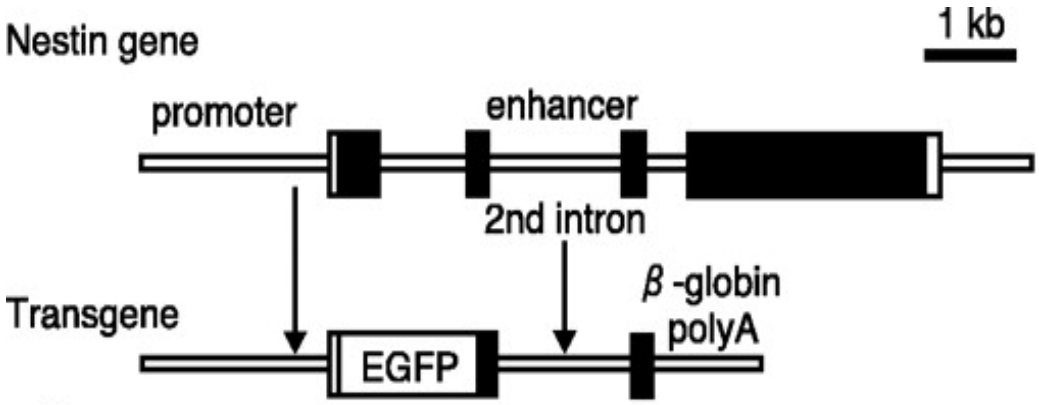

Fig.2.1. Schematic representation of the transgene under control of the rat nestin gene promoter and enhancer. Cited from: M. Yamaguchi, H. Saito, M. Suzuki and K. Mori, Visualization of neurogenesis in the central nervous system using nestin promoter-GFP transgenic mice. NeuroReport 11 (2000), pp. 1991–1996.

2.2. Preparation of acute brain slices

For acute brain slice preparations, nestin-GFP transgenic mice of different ages (8-32 weeks) were decapitated and the brain was immediately removed. Coronal sections of 150 µm were cut from the forebrain using the Leica VT 1000S vibratome (Leica, Heidelberg, Germany). Slice preparation was performed in ice-cold bicarbonate-buffered bath solution gassed with carbogene (5 % CO2 and 95 % O2). The extracellular solution contained: 134 mM NaCl, 2.5 mM KCl, 1.3 mM MgCl2, 2 mM CaCl2, 1.25 mM K2HPO4, 26 mM NaHCO3, 10 mM D-glucose. The brain slices were gently transferred by pipette to a holding chamber and kept in the bath solution at room temperature at least for 60 minutes until used for recording.

2.3. Identification of the nestin-GFP-expressing cells in the subgranular zone and CA1 of hippocampus, corpus callosum, striatum and amygdala

The subgranular zone of hippocampus, CA1 of hippocampus, corpus callosum, striatum and amygdala were recognized, respectively, using transmission light (Zeiss Axioskop, Carl Zeiss, Jena, Germany; 5x Zeiss objective, numerical aperture 0.15) and images were captured with a high-sensitivity CCD camera Variocam (PCO Computer Optics, Kelheim, Germany)(Fig.2.2). The confirmations of the brain slice for the individual region were obtained from web page of the Mouse Brain Library: http://www.mbl.org

Images of nestin-GFP fluorescent cells were obtained by excitation at 480 nm using a monochromator (Polychrome IV, Till Photonics, Martinsried, Germany). The emitted light was collected at 530 ± 10 nm with a CCD camera QuantiCam (b/w VGA, Phase, Lübeck, Germany; 40x water immersion Olympus objective, numerical aperture 0.8). Images were processed with Imaging Workbench (Axon Instruments, Union City, USA). The selected cells were located about 10 to 20 µm below the surface of the slice.

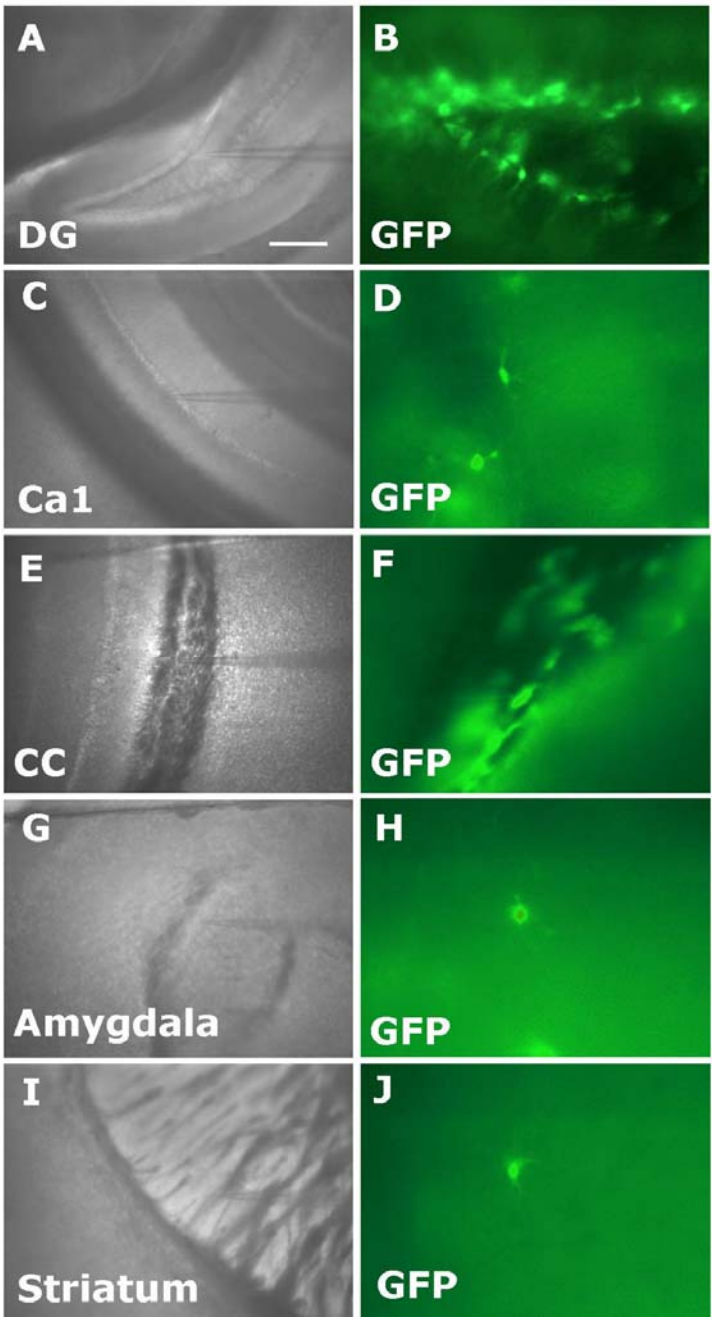

Fig.2.2. Identification of the nestin-GFP-expressing cells in the DG and CA1 of hippocampus, corpus callosum, striatum and amygdala from acute slice. A, C, E, G, I illustrate the coronal section brain slice for the individual region: DG, CA1, CC (corpus callosum), Amygdala and Striatum, respectively. The images were obtained using transmission light (5x Zeiss objective), and images were captured with a high-sensitivity CCD camera. B, D, F, H, J, represent the fluorescence image of GFP-positive cells (excitation at 490 nm and emission at 535 nm) (40x objective) correspond to A, C, E, G, I, using a monochromator. Images were processed with Axon Imaging Workbench. Scale bar denotes 240 μm in A, 30 μm in B, C, and D.

2.4. Materials

The standard bath solution contained 134 mM NaCl, 2.5 mM KCl, 2 mM CaCl2, 1.3mM MgCl2, 26 mM NaHCO3, 1.25 mM K2HPO4, 10 mM glucose. By gassing the solutions with 5% CO2 and 95% oxygen, the pH was adjusted to 7.4–7.5.

The pipette solution contained: 130 mM KCl, 2 mM MgCl2, 0.5 mM CaCl2, 2 mM Na-ATP, 5 mM EGTA, 10 mM HEPES. The pH was adjusted to 7.3 with KOH. All experiments were carried out at room temperature 21-25°C.

Recording pipettes with a resistance of 5–7 MΩ were pulled from borosilicate capillaries (inner diameter 0.87 mm; outer diameter 1.5 mm; Hilgenberg, Malsfeld, Germany) using a P-2000 laser-based pipette puller (Sutter Instrument, Novato, USA).

Slices were superfused with gassed bicarbonate buffer and substances were applied by changing the perfusate. To minimize the indirect neuronal effects induced by the kainate application, 0.5 μM tetrodotoxin (TTX) and 0.1 mM CdCl2 was added to the bath solution to block voltage-gated sodium and calcium channels, respectively. During D-aspartate application, 0.5 μM TTX, 0.1 mM CdCl2 and 50 μM 6-cyano-7-nitro-quinoxaline-2, 3-dione (CNQX), the AMPA/kainate receptors blocker, were added to bath solution. Chemicals were obtained from Sigma (Deisenhofen, Germany) if not otherwise indicated.

2.5. Electrophysiological recordings

2.5.1. Establishing whole-cell recording configuration

Membrane currents were measured with the patch-clamp technique in the whole-cell recording configuration (Hamill et al., 1981) (Fig.2.3). Details see the figure legend of Fig.2.3, which was modified few words from Hamill et al (1981).

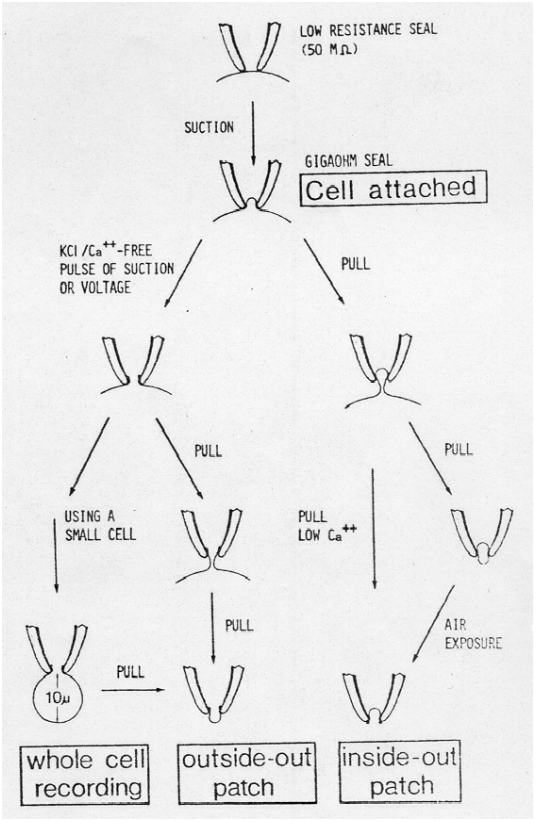

Fig.2.3. Schematic representation of the procedures, which lead to recording configuration. The four recording configurations, described in (Hamill et al., 1981) are "cell-attach", "whole-cell recording", "outside out patch" and "inside-out patch". The upper most frame is the configuration of a pipette in simple mechanical contact with a cell as has been used in the past for single channel recording (Neher et al.1978). Upon slight suction the seal, between membrane and pipette increase in resistance by 2 or 3 orders of magnitude. Forming what we call a cell-attached patch. The improved seal allows a 10-fold reduction in background noise. This stage is the starting point for manipulation to isolate membrane patched which lead to two different cell-free recording configurations ("outside-out patch" and "inside-out patch"). Alternatively, voltage clamp currents from from the cell (pull), short exposure of the pipette tip to air and short pulses of suction or voltage applied to the pipette interior while cell-attached. The figure legend modified from: Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 391, 85-100.

Whole-cell voltage-clamped recordings were obtained from the fluorescence-labeled cells using an EPC 9/2 double patch clamp amplifier in combination with the TIDA software developed in our laboratory.

For the measurement of the whole cell currents, the cell was clamped to a series of de- and hyperpolarizing potentials from –210 mV to +80 mV, or from –140 mV to +20 mV at holding potential of –70 mV, respectively (Fig. 2. 4, A and B).

2.5.2. Study of conductance change induced by neurotransmitter during whole cell recording

To study the conductance change and determine the reversal potential of the response currents induced by different neurotransmitters. The membrane was clamped to a series of de- and hyperpolarizing potential ranging from –130 to 90 mV from a holding potential of –70 mV. These series of voltage jumps was repetitively applied and allowed us to study the conductance changes over time and construct current voltage curves in 6 s intervals (Borges et al., 1994) (Fig.2.4C).

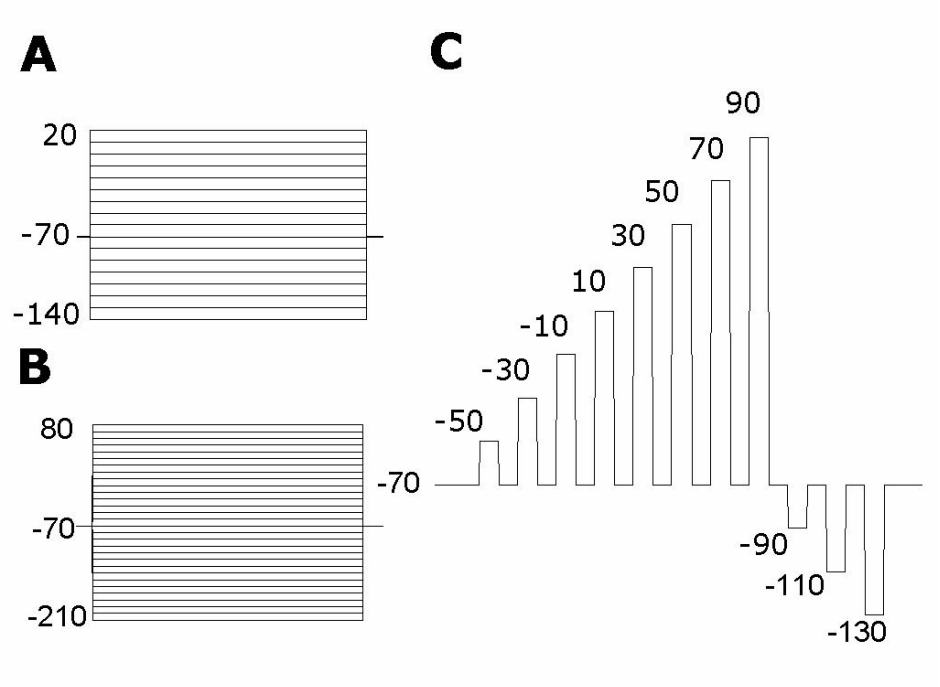

Fig.2.4. Whole cell currents recording protocol. A and B: at holding potential of –70 mV, the cell was clamped to a series of de- and hyperpolarizing potentials from –210 mV to +80 mV, or from –140 mV to +20 mV, respectively. C: Recording protocol was applied to study the conductance change and determine the reversal potential of the response currents induced by different neurotransmitters. Staring from a holding potential of –70mV, the membrane was repetitively clamped to –50, -30, -10, 10, 30, 50, 70, 90, -90, -110, -130 mV for 100 ms separated by 100ms intervals. This stimulation sequence was repetitively applied every 6 s, allowed us to study the conductance changes over time and construct current voltage curves.

2.5.3. Synaptic currents recording

To test for evoked synaptic currents, afferent fibers in the perforant path were stimulated with glass-insulated bipolar platinum wire electrodes (tip diameter 50 µm, tip separation 100-200 µm) (intensity: 1-5 V; duration: 0.2 ms). The stimulated pipette was at least of 150 µm distant from the recorded cell. For the stimulation experiments, 2 mM lidocaine N-ethyl bromide (QX-314) was included in the pipette solution.

2.5.4. Fluorescent dye dialyzed nestin-GFP-expressing cells during whole cell recording

The dentate gyrus of the hippocampus was identified by standard transmission optics (Zeiss Axioskop, Zeiss, Oberkochen, Germany; 5x Zeiss objective, numerical aperture 0.15) and images could be recorded with a CCD camera and stored on computer (Variocam, PCO Computer Optics, Kelheim, Germany). Cells located about 10-30 µm below the surface of the slice were visible with water immersion optics using a 510/20-nm band pass filter for enhanced green fluorescent protein fluorescence detection (excitation max, 490 nm) and photographed using a CCD camera (Watek Instruments) for recording cell size, location and morphology.

To label the recorded cells, Alexa Fluor 594 (10µg/ml, Molecular Probes) was added to the pipette solution. Nestin-GFP-expressing cells were identified by fluorescence optics (excitation at 488 nm using a monochromator, Polychrome IV, Till Photonics, Martinsried, Germany). The emitted light was collected at 530 ± 10 nm with a CCD camera (QuantiCam Phase, Lübeck, Germany; 60x water immersion Olympus objective, numerical aperture 0.8). After the patch clamp recording, the Alexa Fluor 594 fluorescence was detected at an excitation wavelength of 589 nm and an emission at 616 ± 4 nm.

3. Results

3.1. Two distinct subpopulations of nestin-expressing progenitor cells were distinguishable in adult mouse dentate gyrus

Usage of transgenic mice expressing GFP under the promoter for nestin, an intermediate filament present in progenitor cells, combined of immunohistochemistry, electron microscopy and electrophysiology approach, we have demonstrated two distinct subpopulations of nestin-GFP-expressing cells can be distinguished by the morphological features. We named tape1 cells, which had an elaborate tree of processes in the granule cell layer and expression of GFAP. Electron microscopy revealed vascular end feet of nestin-positive cells, which supporting astrocytic differentiation. Electrophysiological feature of nestin-GFP-expressing cells on acutely isolated hippocampal slices showed passive current characteristics of astrocytes in type1 cells. Another subpopulations of the nestin-GFP-expressing cells, named type2 cells, which lacks astrocytic features, with the electrophysiological feature of delayed-rectifying potassium currents and a very small number of cells with sodium currents. Details See (Filippov et al., 2003).

Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. Filippov V, Kronenberg G, Pivneva T, Reuter K, Steiner B, ***Wang LP****, Yamaguchi M, Kettenmann H, Kempermann G. Mol Cell Neurosci. 2003 Jul; 23(3): 373-82.*

3.2. Earliest synaptic input during adult hippocampal neurogenesis is GABAergic

3.2.1. The non-radial precursor cells can express neuronal properties

Using the transgenic nestin-GFP animals, precursor cells could be recognized in acute slices of hippocampus based on their GFP-fluorescence. As previously reported, we could distinguish two types of precursor cells based on morphological criteria and their location

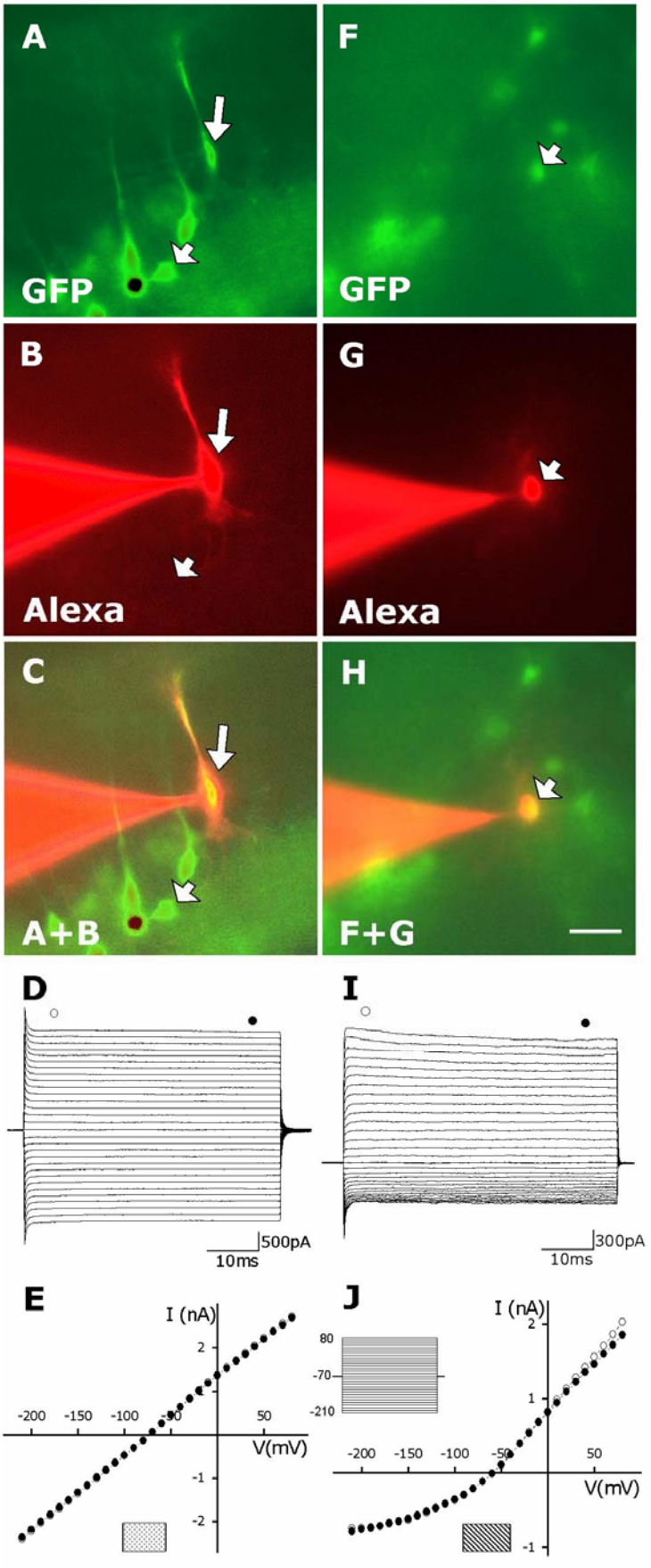
A
GFP
F
GFP
B
Alexa
G
Alexa
C
A+B
H
F+G
D
500pA
10ms
I
300pA
10ms
E
I (nA)
2
-1
-2
-200
-150
-100
-50
50
V(mV)
J
I (nA)
2
1
-1
80
-70
-210
-200
-150
-100
-50
50
V(mV)

Fig.3.1. Morphology and current pattern of a type-1 (A-E) and type-2 precursor cell (F-J) in the dentate gyrus from adult nestin promoter-GFP mice

A: Fluorescence image of GFP-positive cells (excitation at 490 nm and emission at 535 nm). The cell marked by arrow was recorded from and dialyzed with Alexa Fluro 594 via the pipette solution. B shows the Alexa Fluro 594 staining (excitation at 590 nm and emission at 645 nm). C: The merged image verifies that the GFP and Alexa Fluro 594 labels correspond. D: Membrane currents from the cell shown in A-C were evoked by 50 msec voltage steps ranging from –210 mV to +80 mV from holding potential of –70 mV (the recording protocol see the inset in J). E: The current to voltage curve was obtained from D; current amplitudes at the beginning (open circle) and at the end of the voltage pulse (black circle) were plotted against the holding potential. F-J: Similar as described for A-E, a type-2 cell was characterized. Note the lack of processes particularly evident in G, and the outward rectification of the current voltage curve. Scale bar denotes 25 μm.

in the subgranular zone. Type-1 cells had one thick process extending into the granule cells layer and processes branching extensively in the inner molecular layer. In contrast, type-2 cells showed only plump horizontal processes or lacked processes. We dialyzed cells with the hydrophilic fluorescent dye Alexa Fluor (10 μg/ml) via the patch-pipette to document the morphological features and to distinguish between type-1 and type-2 cells (Fig.3.1, A and C; F and H respectively). After establishing a whole-cell recording configuration, the cell was clamped to a series of de- and hyperpolarizing potentials from –210 mV to +80 mV or from –140 mV to +20 mV at holding potential of –70 mV. Almost all type-1 cells were characterized by passive membrane currents with no apparent time-dependence and a linear current to voltage curve (95 %; 77 out of 81 cells) (Fig.3. 1, D and E). The remainder (4 out of 81) showed an outward rectification similar to the recording obtained from a type-2 cells shown in Figure 1 (I and J). The outward current slightly inactivated, but there was no apparent time-dependent activation. Among the type-2 cells, half were characterized by passive currents (51.5 %; 35 out of a total of 78 cells), about another population (12 cells, 17.6 %) showed the outward rectification (Fig.3. 1, I and G) similar as the currents described for the type-1 cells. 7 cells of the type-2 cells showed delayed rectifying currents with depolarization and inward rectifying, inactivating currents with hyperpolarization (Fig.3. 2, A). Five of the type-2 cells (7.4 %) exhibited small rapidly inactivating inward currents with depolarization typical for voltage-gated Na^+ channels followed by delayed outward currents. With hyperpolarization, no currents were activated (Fig.3. 2, B). To test for the ability of these cells to generate actions potentials, we injected depolarizing current in the current clamp mode, but failed to record action potentials (Fig.3. 2, D). These cells typically had a high input resistance (range from 600 to 5100 MΩ). The last group (9 out of 78 cells, 13.2 %) with type-2 morphology was characterized by the

presence of large inward currents typical for Na^+ currents of neurons. In the current clamp mode, action potentials could be recorded after depolarizing current injection (Fig.3. 2, E). We conclude that the radial cells are predominantly characterized by passive membrane conductance, while the type-2 cells are more heterogeneous including cells with neuronal properties.

3.2.2. Stimulation of the perforant path triggers GABAergic synaptic currents in a small population of the non-radial precursors

To test for synaptic input to nestin-GFP-expressing cells, we placed an extracellular stimulation electrode at the perforant path, while recording membrane currents from type-2 cells. In six cells we recorded transient inward currents in response to a 0.2 ms stimulation pulse. In all these cells, we recorded large Na^+ inward currents briefly after establishing the whole cell recording configuration. These currents rapidly disappeared since, in this set of experiments, we used lidocaine in the patch pipette to block Na^+ currents after dialysis of the cell (Fig.3. 3, A and D). The stimulus-evoked currents had amplitude from 108 to 863 pA and decayed in average within 48 ms (range 15 to 110 ms). Clamping the membrane potential to a series of de- and hyperpolarizing potentials allowed to construct the current voltage curve of the stimulus evoked current. The response was inward rectifying and reversed close to 0 mV (Fig.3.3, E and F). We also observed spontaneous synaptic activity. The rise time of these spontaneous currents was about 1.5 ms and the decay time constant (as determined by monoexponetial fitting) ranged between 5 and 93 ms (20.1 ± 1.99 (mean ± SE), n=63) with peak amplitudes ranging from 30 to 140 pA. The spontaneous and the stimulation-induced activity could be irreversibly blocked by tetrodotoxin (n=4; 0.5 μM, Fig.3. 4, A and B) or reversibly by bicuculline (n=4; 20 μM) (Fig.3.5, C-E) indicating that precursor cells receive neuronal GABAergic input. In contrast, the kainate/AMPA receptors blocker CNQX (50 μM) and NMDA receptor blocker APV (n=4; 50 μM) did not affect the rate of spontaneous activity (Fig.3.5, F). Bicuculline, CNQX and APV were all tested on the same cells.

In additional 13 cells, stimulation triggered an inward current with amplitude ranging between 35 to 106 pA. 11 out of 13 cells were type-2 cells, 2 cells were type-1. When clamping the membrane to a series of potential ranging from –100 to +20 mV, the current

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amplitude was not affected. Application of tetrodotoxin reversibly blocked this response. We did not analyze this response further.

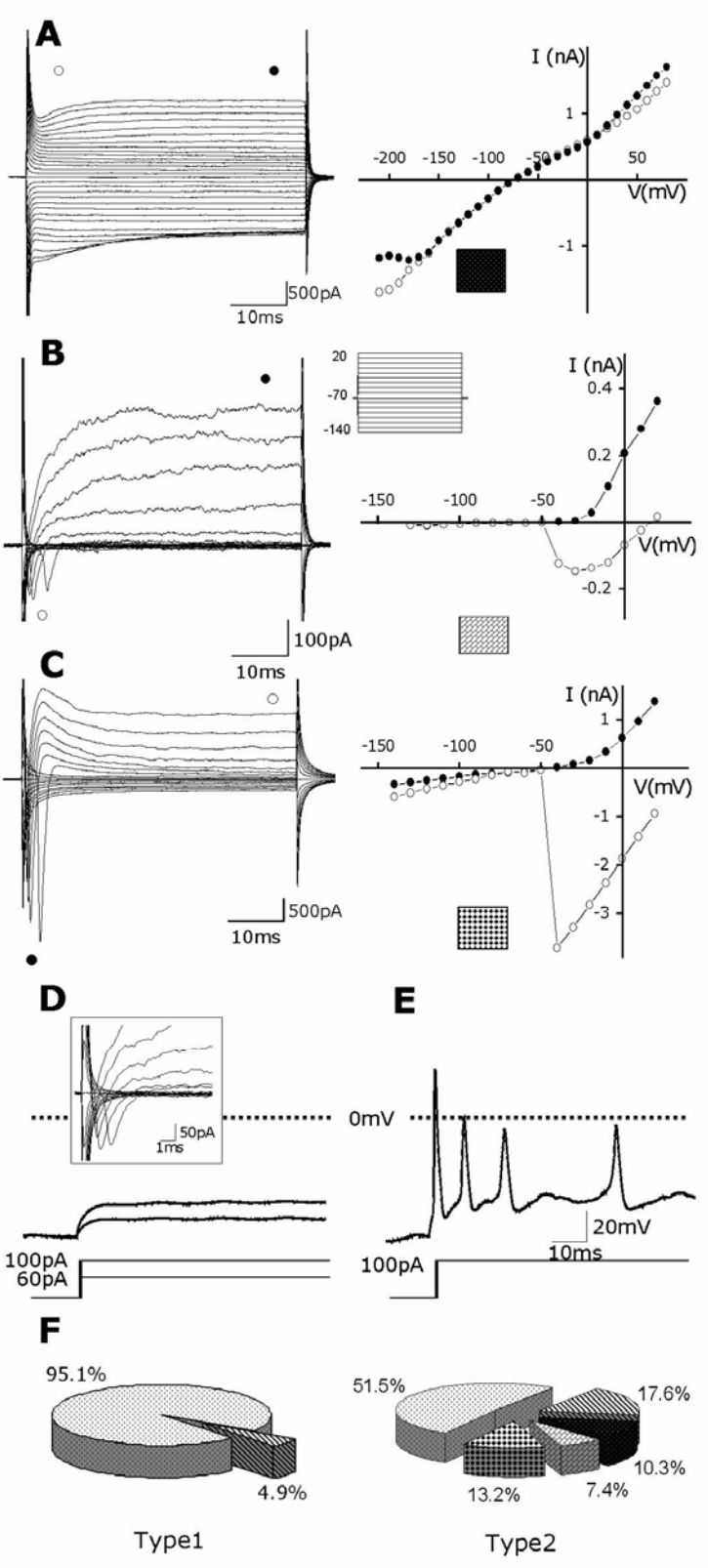


Fig.3.2. Heterogeneous current pattern in the type-2 cells

In A-C, membrane currents were activated similar as described in the legend to Fig.3.1 and the resulting current voltage curves are depicted on the right. Current amplitudes at the beginning (open circle) and at the end of the voltage pulse (black circle) were plotted against the holding potential. In B and C, the voltage jumps were restricted from –140 to +20 (the recording protocol see the inset in B). Note the small transient inward current in B, the large ones in C. D: To test for the ability of the cell shown in B to generate action potentials, current pulses (100 pA and 60 pA) were injected. The depolarization did not trigger an action potential. The transient inward currents were magnified in the inset. E: Action potentials were recorded in response to depolarizing current injection from the cell shown in C. F: Distribution of membrane current pattern in type-1 (left) and type-2 precursor cells (right). The shading corresponds to the insets in the five examples shown in Fig.3. 1. D-E and I-J and Fig.3. 2, A-C.

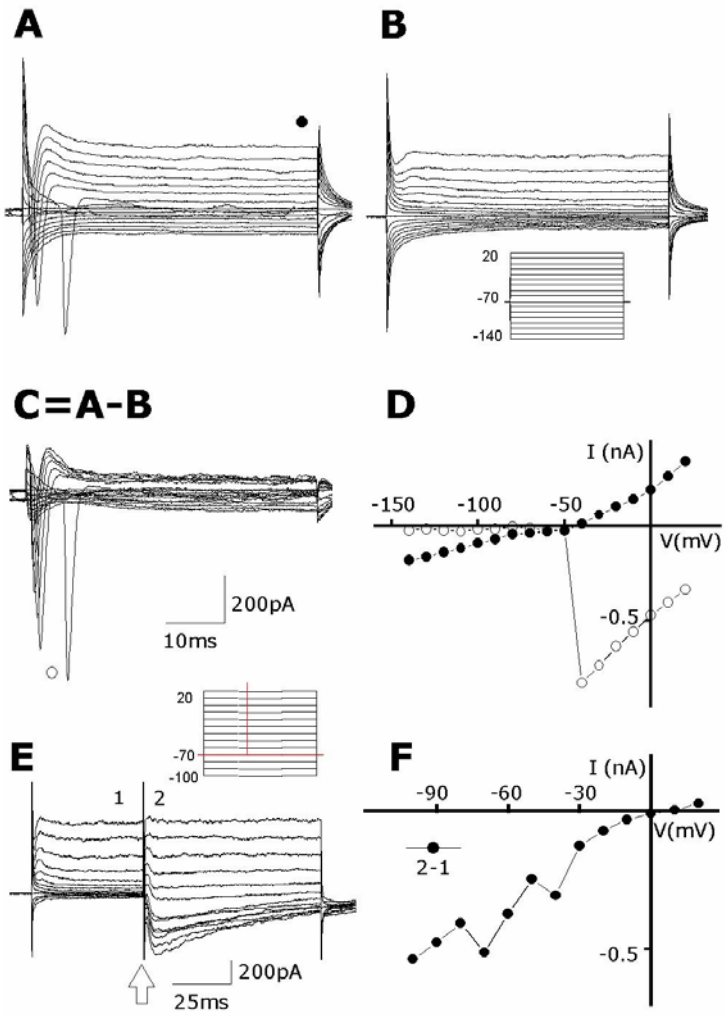


Fig.3.3. Synaptic current recordings in a cell with large Na^+ currents.

A: Briefly after establishing the whole cell recording mode, membrane currents were activated as described in the legend to Fig.3. 2, B from a type-2 precursor cell. Note the large transient inward currents. B: 1 min after dialysis of the cell with pipette solution containing the Na^+ channel blocker lidocaine (2 mM), the transient inward current was no longer recorded. To isolate the Na^+ current trace B was subtracted from trace A and the result is displayed in C. In D, current voltage curves from currents shown in A measured at a time point indicated by black circle and from C by open circle are displayed. The current voltage curve displayed in black circles has the typical course as described for delayed rectifying K^+ channels, the one displayed in open circles as described for voltage gated Na^+ channels. E: The membrane was clamped at a series of potentials for 130 ms and after 50 ms, a brief stimulation pulse was applied to the perforant path (arrow) (the recording protocol see the inset). To isolate the stimulus induced currents amplitudes, currents just prior to the stimulus (1) and briefly after (2) were subtracted and the resulting current voltage curve is displayed in F.

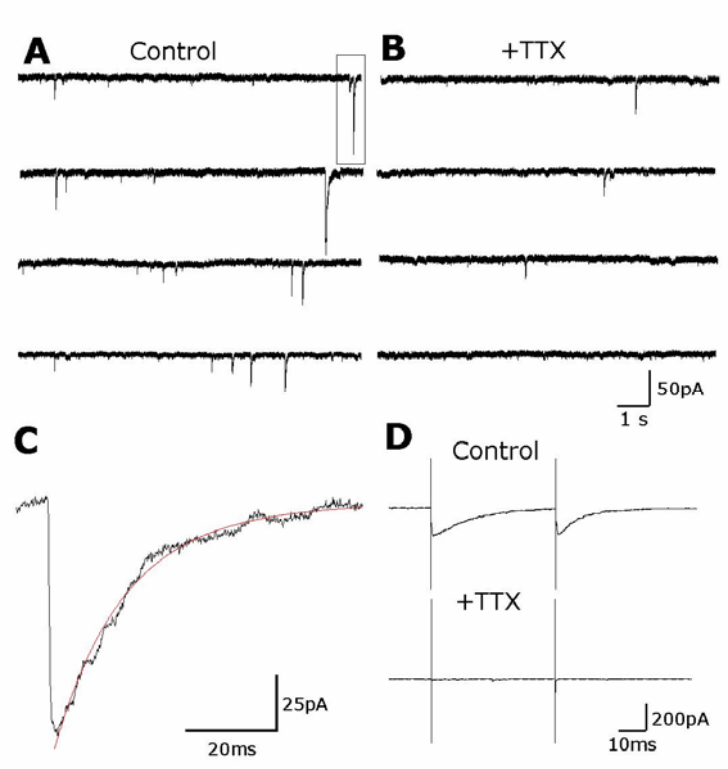


Fig.3.4. Spontaneous and evoked activity and its sensitivity to TTX

A, B Membrane currents were recorded from a type-2 precursor cell at a holding potential of –70 mV. Note the spontaneous transient inward currents. The four traces are examples from ten subsequently recorded traces showing those with the most activity. In B, TTX (0.5 µM) was washed in. C: A single spontaneous event outlined in A by square is shown in expanded time scale. The monoexponetial fit is superimposed yielding a time constant of 20 msec; D: Currents evoked by paired-pulse stimulation (0.2 msec with 50 msec interval) of the perforant path in absence and presence of TTX (0.5 µM). Transients before the traces are stimulus artifacts. The membrane was clamped at -70 mV.

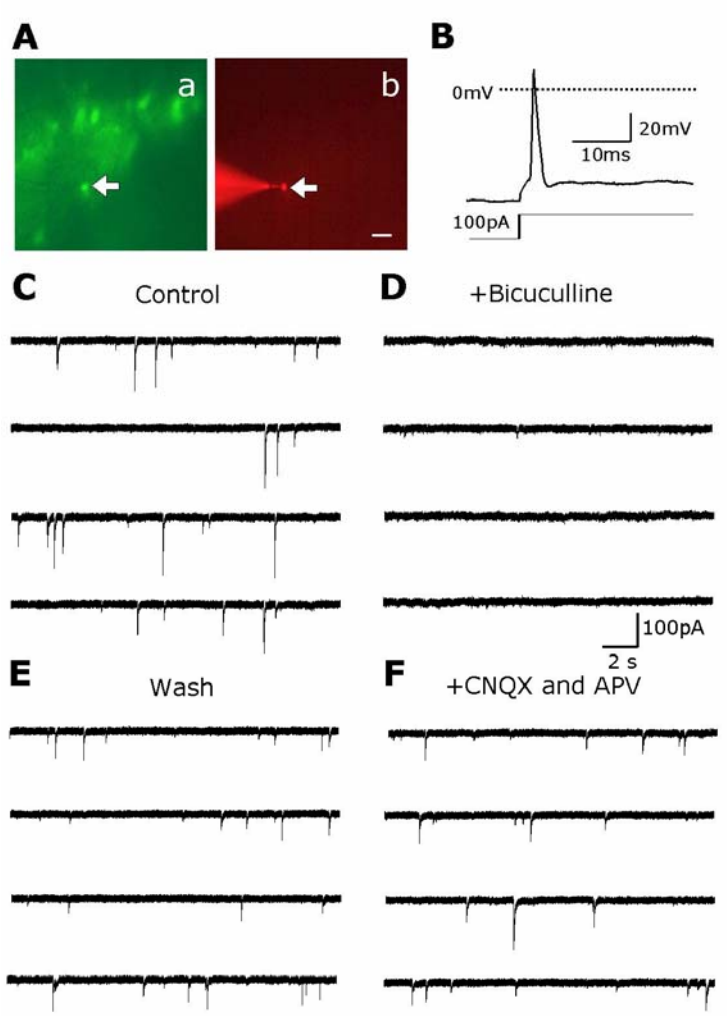


Fig.3.5. Spontaneous activity is sensitive to bicuculline, but not to CNQX and APV

A: Similar as described in the legend to Fig.3.1, the GFP (left) and Alexa Fluro 594 fluorescence (right) is displayed in the two images from a type-2 cell. Scale bar denotes 25 μm. B: From the same cell an action potential was recorded in the current clamp mode in response to a current injection (100 pA, 30 msec) C-F: Spontaneous activity was recorded in control conditions (C), after wash in of bicuculline (20 μM, D), after washout of bicuculline (E) and after co-application of CNQX (50 μM) and APV (50 μM).

3.2.3. Both types of nestin-GFP-expressing cells respond to GABA

To study the functional expression of GABAA receptors, we tested the effect of GABA and the GABAA receptor agonist muscimol on membrane currents of nestin-GFP-positive cells. Bath application of GABA (0.5 mM, 30 sec) while clamping the membrane at –70 mV, elicited an inward current of 17 pA to 794 pA (Mean, 119 pA; n=35,Fig.3.6, A), muscimol 49 pA to 65 pA (Mean, 58 pA; n=4). 68.5 % of type-1 cells (24 out of 35) and 84.6 % of type-2 cells (11 out of 13) responded to GABA or muscimol application. To determine the reversal potential of the GABA response, the membrane was clamped to a series of de- and hyperpolarizing potential ranging from –130 to 30 mV from a holding potential of –70 mV. These series of voltage jumps was repetitively applied and allowed us to study the conductance changes over time and construct current voltage curves in 6 s intervals. The current voltage curve of GABA induced currents was obtained by subtracting the current measured before the application of the GABA from that observed at the peak of the GABA response. The current voltage curve showed a slight inward rectification at positive potentials indicating that outward K^+ currents were blocked similar as described for granule cells of the hippocampus (Labrakakis et al., 1997). The reversal potential was close to 0 mV when determined by extrapolation from currents between –130 to –50 mV thus close to the chloride equilibrium potential (Fig.3.6, C). The GABAA receptor antagonist bicuculline reversibly blocked the GABA response (n=4, data not shown).

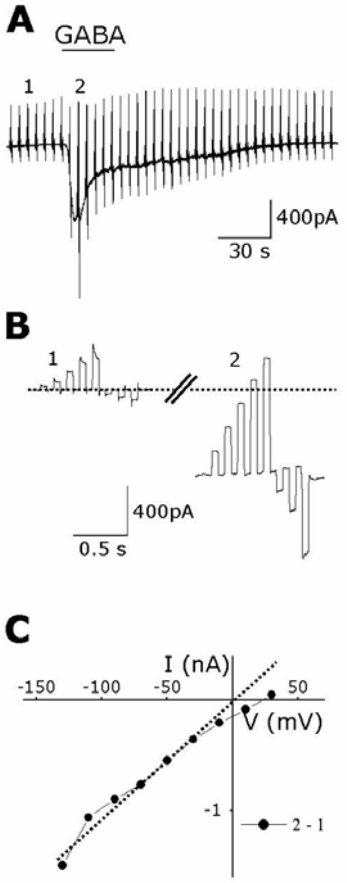
3.2.4. Nestin-GFP-expressing cells express functional glutamate receptors of the AMPA subtype

To study the functional expression of AMPA/kainate glutamate receptors, we tested the effect of kainate on membrane currents of nestin-GFP-positive cells. To minimize the indirect effects from neurons, 0.5 µM TTX and 0.1 mM CdCl2 was added to the bath solution to block voltage-gated sodium and calcium channels, respectively. Bath application of kainate (0.5 mM, 30 sec) triggered an inward current while clamping the membrane at –70 mV; the current attitudes in type-1 cells averaged at 155.9 ± 28.6 pA (mean ± SE, n=32 responding cells out of 37 tested), in type-2 cells at 257.9 ± 53.6 pA (mean ± SE, n=19 responding cells out of 22 tested) (Fig.3.7, A).

To distinguish between AMPA and kainate receptors, we used cyclothiazide, which selectively promotes currents through AMPA receptors by blocking receptor desensitization, while keeping kainate receptors unchanged (Partin et al., 1993; Patneau et al., 1993; Yamada and Tang, 1993).

Fig.3.6. Functional expression of GABA receptors

A: During recording of membrane currents from a type-2 cell, GABA (1 mM, 30 sec) was applied as indicated. The membrane was clamped from a holding potential of -70 mV to a series of de- and hyperpolarizing potentials ranging between -130 mV to +30 mV with 20 mV increments. At this time resolution, the single voltage steps can not be resolved B: Two series of voltage steps from the recording shown in (A) before (1) and at the peak of the GABA response (2) are shown with higher time resolution and were used to construct a current voltage curve as shown C: The current voltage curves were obtained by subtracting current amplitudes at the corresponding membrane potentials before and during the GABA application. The reversal potential was close to 0 mV when determined by extrapolation from currents between –130 to –50 mV thus close to the chloride equilibrium potential.



The currents induced by kainate were increased by 59 % to 690 %, when coapplying cyclothiazide (100 µM, 30 sec, n=7 out of 7 tested) (Fig.3.7, B). To obtain the current voltage curve of the kainate response, the membrane was clamped to a series of de- and hyperpolarizing potential ranging from –130 to 90 mV from a holding potential of –70 mV. (We used the same paradigm as described above for GABA currents.) The current voltage curve was linear with a reversal potential at + 6.1 mV. A similar current voltage curve, yet with a larger conductance, was obtained in the presence of cyclothiazide (reversal potential –3.5 mV) (Fig.3.7, D). We conclude that both types of precursor cells express AMPA receptors.

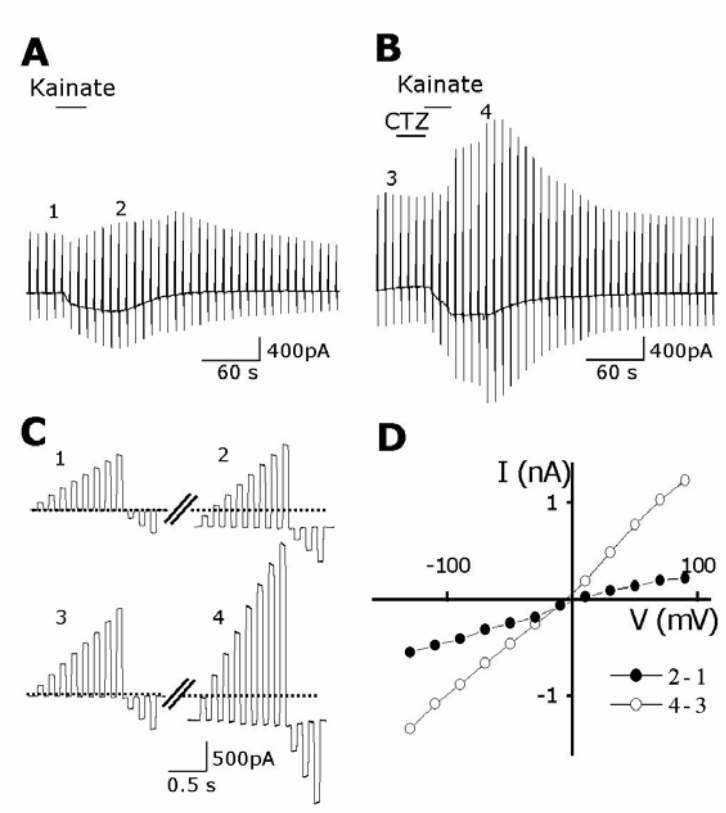


Fig.3.7. Identification of AMPA/kainate receptor

A: Similar as described in the legend to Fig.3.6, kainate (0.5 mM, 30 sec) was applied while recording membrane currents from a type-2 cell. B: After wash, the same cell was incubated with 100 μM CTZ (30 sec) and then kainate was applied. Note the increase in membrane currents as compared to the recording in A. C: Two series of voltage steps from the recording shown in A (top) and B (bottom) before (1) and at the peak of the kainate response (2) are displayed in higher time resolution and were used to construct a current voltage curve as shown D: The current voltage curves were obtained by subtracting current amplitudes at the corresponding membrane potentials before and during the kainate application with (open circles) and without pre-application of CTZ (black circles).

3.2.5. Nestin-expressing precursor cells express functional glutamate transporters

Because nestin-expressing precursor cells share features with astrocytes, we tested for the presence of glutamate transporter currents by applying D-aspartate, a substrate of the glutamate transporter. 0.5 μM TTX, 0.1 mM CdCl2 and 50 μM 6-cyano-7-nitro-quinoxaline-2, 3-dione (CNQX), the AMPA/kainate glutamate subtype receptors blocker,

were added to the bath solution to minimize interfering effects from neurons or by kainate/AMPA receptor activity. Similar as for analyzing GABA or kainate-induced currents, we clamped the membrane to a series of potentials between -130 and +90 mV. The resulting current to voltage curve of D-aspartate-induced currents was characterized by lacking reversal and prominent inward rectification. (Fig.3.8). In type-1 cells D-aspartate evoked an inward current of 72.3 ± 12.3 pA (mean ± SE, n = 20 responding cells out of 25 tested), in type-2 cells of 65.7 ± 13.0 pA (mean ± SE, n = 11 responding cells out of 16 tested) at a holding potential of –70 mV.

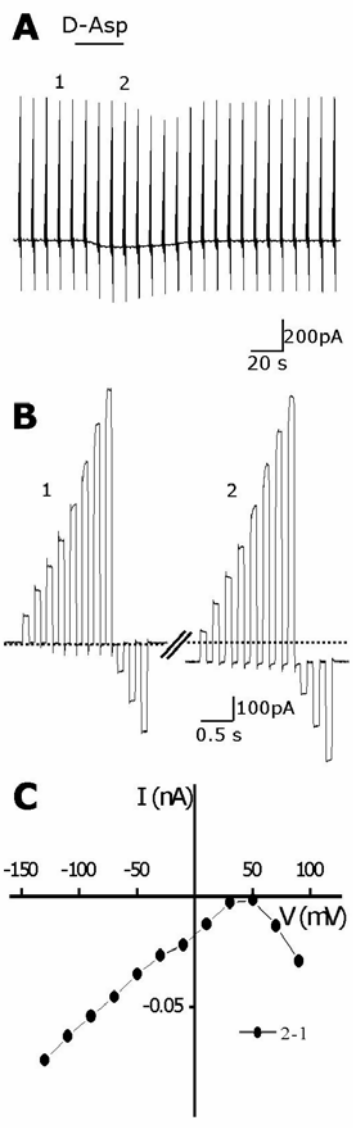


Fig.3.8. Functional expression of glutamate transporter currents

A: Similar as described in the legend to Fig. 6, the current response to D-Aspartate (0.5 mM, 30 sec) was analyzed from a type-1 cell. B: Two series of voltage steps from the recording shown in (A) before (1) and at the peak of the D-Aspartate response (2) were used to construct a current voltage curve as shown C: The I-V curves of D-Aspartate evoked current was characterized by lacking reversal and prominent inward rectification.

3.3. Functional expression of the nucleotide receptors by nestin-expressing precursor cells in the adult murine hippocampus

In order to investigate whether progenitor cells of the dentate gyrus respond to extracellular nucleotides we analyzed progenitor cells in mice transgenic for GFP under the control of the nestin promoter. Cells could be identified in acutely isolated hippocampal slices by their green fluorescent label. We have previously shown that nestin-expressing precursor cells in the adult subgranular zone of hippocampal dentate gyrus can be divided into two distinct subpopulations based on morphological criteria (Filippov et al., 2003). Type-1 cells expressed glial fibrillary acidic protein (GFAP), and were characterized by long radial-glia-like processes spanning the entire granule cell layer. Type 2 cells had shorter horizontal or no processes and lacked

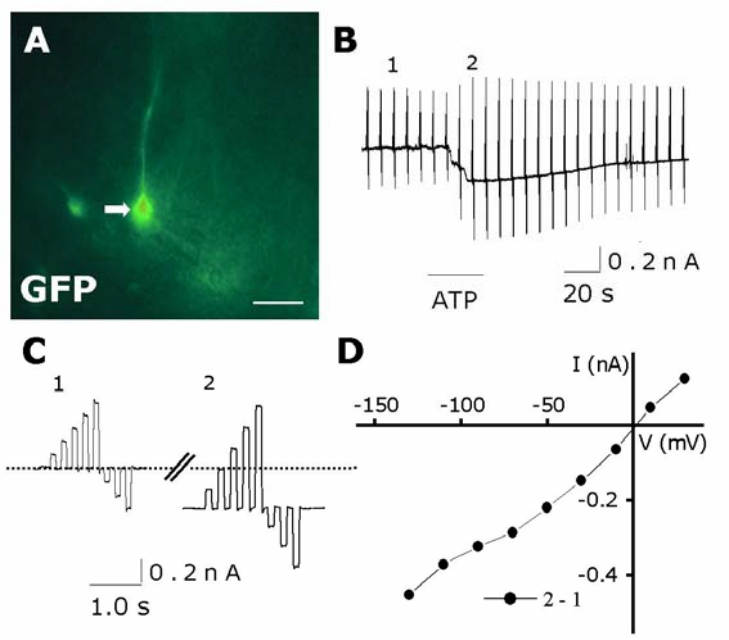


Fig. 3.9. Response to extracellular ATP in GFP-expressing precursor cells.
A: A micrograph showing the fluorescence image of a green fluorescent protein-labeled cell in the dentate gyrus of the hippocampal slice. The recording shown in B and C was obtained from this cell. Scale bar = 20 μm. B: The membrane was clamped from a holding potential of -70 mV to a series of de- and hyperpolarizing potentials ranging between -130 mV to +30 mV with 20 mV increment. In this graph the single voltage steps cannot be resolved but it rather reveals the overall change in membrane conductance over time. ATP (1 mM) was applied as indicated. C: Two series of voltage steps from the recording shown in (B) before (1) and at the peak of the ATP response (2) were used to construct a current voltage curve as shown on the right.

expression of GFAP. These cells were approached with the patch pipette to record membrane currents in response to ATP, using the whole cell-recording mode of the patch clamp technique (Fig.3. 9). When the membrane was clamped at -70 mV, ATP elicited an inward current in most radial cells (type-1; 13 out of 18) and in a minority of cells with a horizontal orientation lacking extensive processes (type-2, 2 out of 14). To obtain the reversal potential of the response we repetitively clamped the membrane at a series of de- and hyperpolarizing potentials between -130 mV and +30 mV and this series of voltage steps was repeated every 6 sec. As shown in Fig. 3.9, B, C, a current voltage curve was obtained by subtracting currents at the peak of the ATP response from control currents. The current voltage curve was essentially linear and reversed close to 0 mV. We conclude from these data that a subpopulation of precursor cells expressed ionotropic P2X receptors. In 3 out of 7 cells we also observed small inward currents in response to ADP and UTP. Since these currents were fairly small we did not analyze this response further.

3.4. Membrane current features and functional expression of AMPA/Kainate receptors by nestin-GFP-expressing cells in the adult amygdala

3.4.1. Membrane current features of Nestin-GFP-expressing cells in amygdala

To analyze the physiological properties of precursor cells in the amygdala, slices were prepared from nestin-GFP-transgenic mice. We tested for the expression of voltage-gated channels, AMPA/kainate receptors and glutamate transporters. Nestin-GFP-expressing cells could be recognized due to their green fluorescence and were subsequently approached with the patch pipette (Fig.3.10, A-D). To reveal the morphological features of the recorded cell, the hydrophilic fluorescent dye Alexa Fluor 594 (10 µg/ml) was added to pipette solution and the cell was brightly stained after dialysis. After establishing the whole cell recording configuration (Hamill et al., 1981), in the voltage clamp mode, membrane currents were activated by clamping the membrane at a holding potential of -70 mV to hyper- and depolarizing potentials ranging from - 180 to +80 mV (50 ms, 10 mV increment). We selected for cells with a stable membrane potential between -50 and –85

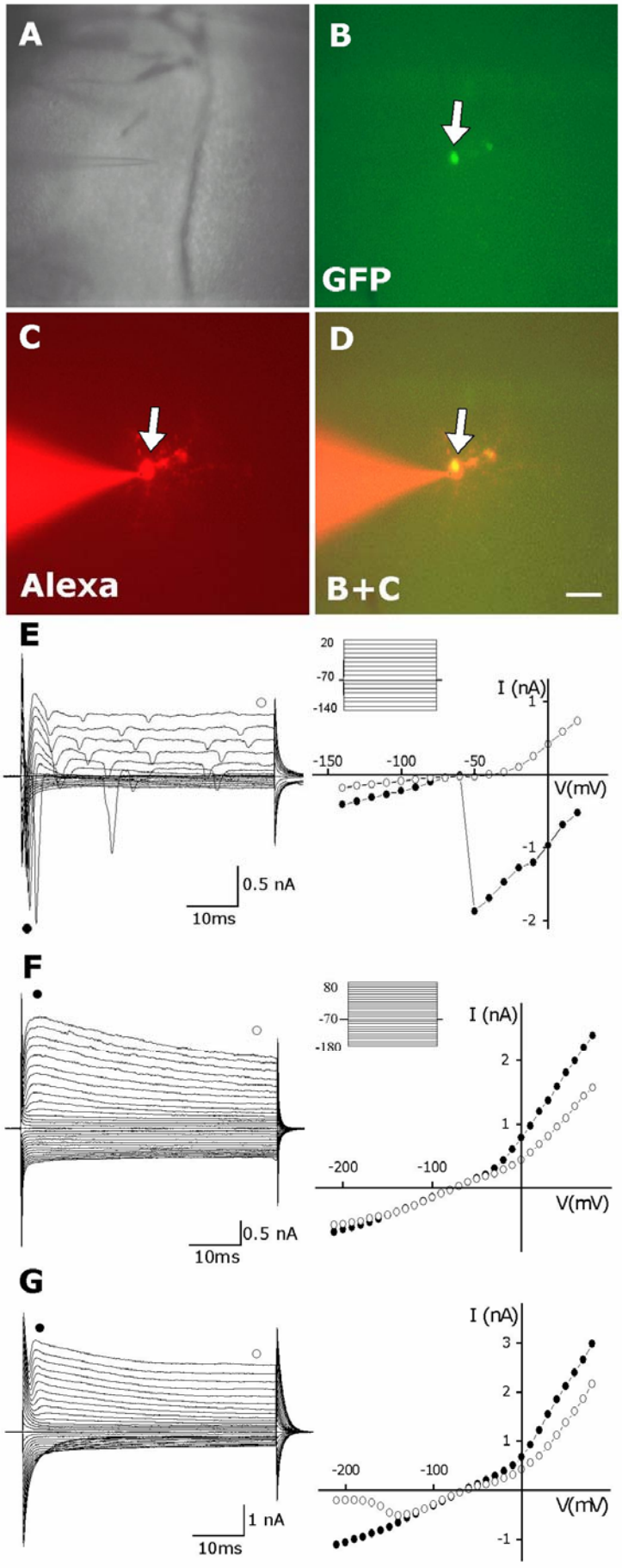

A
B
GFP
C
Alexa
D
B+C
E
20
-70
-140
I (nA)
1
-150
-100
-50
V(mV)
-1
-2
0.5 nA
10ms
F
80
-70
-180
I (nA)
2
1
-200
-100
V(mV)
0.5 nA
10ms
G
I (nA)
3
2
1
-200
-100
V(mV)
-1
1 nA
10ms

Fig. 3.10. Morphology and current pattern of nestin-GFP-expressing cells in the amygdala from adult nestin promoter-GFP transgenic mice

A. Bright-field images of a striatum slice showing the position of the patch pipette during recording. B. Fluorescence image of GFP- expressing cells (excitation at 490 nm and emission at 535 nm). The cell marked by arrow was recorded from and dialyzed with Alexa Fluro 594 via the pipette solution. C. Shows the Alexa Fluro 594 staining (excitation at 590 nm and emission at 645 nm). D. The merged image verifies that the GFP and Alexa Fluro 594 labels correspond. E. Left panel: Membrane currents from the cell shown were evoked by 50 msec voltage steps ranging from –140 mV to +20 mV from holding potential of –70 mV (the recording protocol see the inset in E). Note the transient inward current in E. E. Right panel: voltage curve was obtained from left panel; current amplitudes at the beginning (black circle) and at the end of the voltage pulse (open circle) were plotted against the holding potential. F. Left panel: Membrane currents from the cell shown were evoked by 50 msec voltage steps ranging from –210 mV to +80 mV from holding potential of –70 mV (the recording protocol see the inset in F). Right panel: The outwardly rectifying current to voltage curve was obtained from left panel; current amplitudes at the beginning (black circle) and at the end of the voltage pulse (open circle) were plotted against the holding potential. G: Similar as described for F, another GFP-positive cell was characterized by outwardly rectifying and inward rectifying current voltage curve. Scale bar denotes 120 μm in A, 15 μm in B, C, and D.

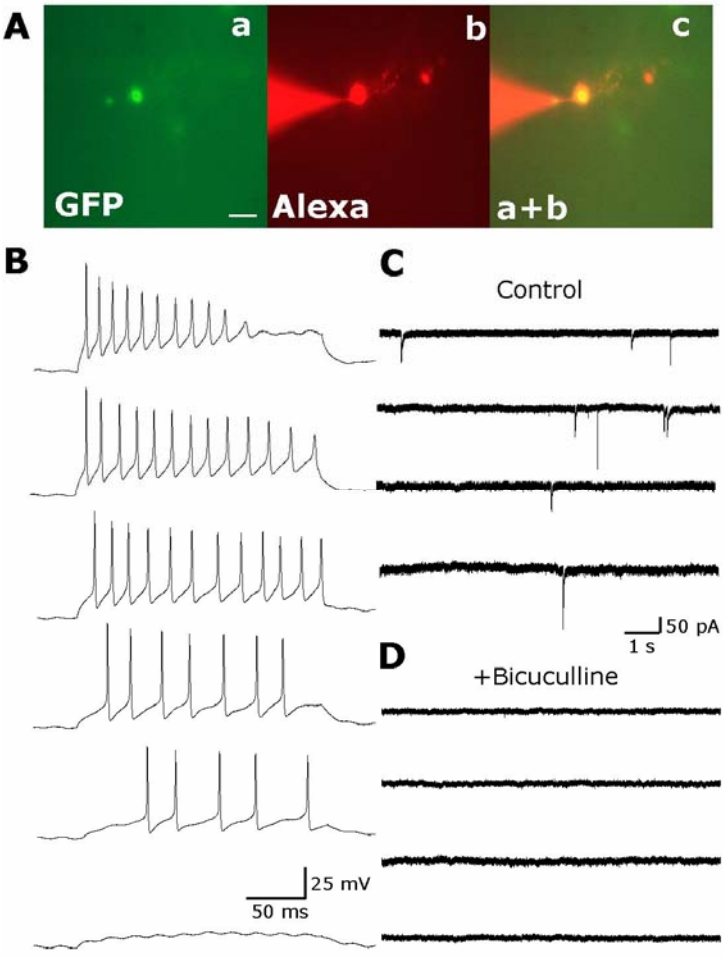


Fig. 3. 11. Neuronal properties were observed in a few nestin-GFP-expressing cells in the amygdala
A. Fluorescence image of a GFP-expressing cells (excitation at 490 nm and emission at 535 nm) in amygdala was recorded the sodium current which was shown in Fig.3.10, E. The GFP image, the Alexa dialyzed image and the merged image of the cell during recording shown in a, b, c, respectively, Scale bar denotes 25 μm. B. In the current clamp mode, action potentials could be recorded after depolarizing current injection, and displayed the depolarizing current-dependents frequency. C and D. Spontaneous activity was recorded in control conditions (C), in the present of bicuculline (20 μM, D).

mV (N=51). Three different patterns of membrane current were distinguishable in nestin-GFP-expressing cells in amygdala. The majority (27 out of 49) of nestin-GFP-expressing cells expressed outwardly rectifying currents activated with depolarization and inwardly rectifying, inactivation currents with hyperpolarization. The current voltage curve revealed a reversal potential close to the K^+ equilibrium potential (Fig.3.10, G). A second population (19 out of 49) of cells we studied displayed the current pattern was characterized by the presence of outwardly rectifying currents with depolarization and voltage- and time-independent currents with hyperpolarization. The current voltage curve revealed outwardly rectifying properties and a reversal potential close to the K^+ equilibrium potential (Fig.3.10, F).
Few cells (3 out of 49) had neuronal properties. Rapidly activating and inactivating inward currents were trigged with depolarization positive to about −50 mV that was typical for voltage-gated Na^+ currents. The inward current was followed by an outward current with a reversal potential close to the K^+ equilibrium potential (Fig.3.10, E). Action potentials could be recorded after depolarizing current injection in the current clamp mode and displayed the depolarizing current-dependents frequency. We also observed spontaneous synaptic activity in the three cells. The rise time of these spontaneous currents was about 1.5 ms and the decay time constant (as determined by monoexponetial fitting) ranged between 7 and 105 ms, n=36) with peak amplitudes ranging from 39 to 154 pA. The spontaneous activity could be blocked by bicuculline (n=3; 20 μM) (Fig.3. 11) indicating that precursor cells receive neuronal GABAergic input.

3.4.2. Nestin-GFP-expressing cells in amygdala express functional glutamate receptors of the AMPA/Kainate subtype in situ

Stem cells in the dentate gyrus express glutamate receptors and transporters while in the CA1 region of the hippocampus astrocytes either express receptors or transporters. We

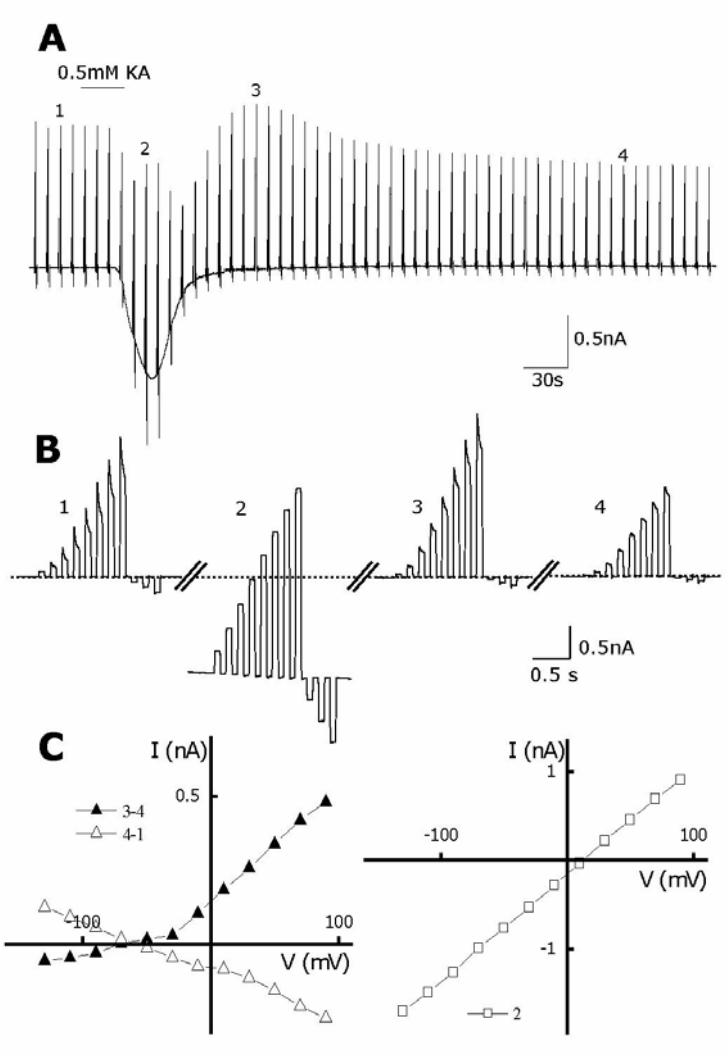


Fig.3.12. Functional expression of AMPA/kainate glutamate receptors in nestin-GFP-expressing cells in the amygdala

A: During recording of membrane currents from a nestin-GFP- expressing cell, Kainate (0.5 mM, 30 sec) was applied as indicated. The membrane was clamped from a holding potential of -70 mV to a series of de- and hyperpolarizing potentials ranging between -130 mV to +30 mV with 20 mV increments. At this time resolution, the single voltage steps cannot be resolved. B: Four series of voltage steps from the recording shown in (A) before (1), at the peak of the kainate response (2), the developed outward rectifying current (3) and long term blockage of the resting K^+ conductance (4), are shown with higher time resolution and were used to construct a current voltage curve as shown C: Left panel: The current voltage curves of long term blockage of the resting K^+ conductance were obtained by subtracting current amplitudes at the corresponding membrane potentials before and about 12 mins after the kainate application (4-1). The reversal potential was close to -80 mV. The current voltage curves of the outward rectifying current were obtained by subtracting current amplitudes at the corresponding membrane potentials in the peak of the outward rectifying current and about 12 mins after the kainate application (3-4). The reversal potential was close to -70 mV. C: Right panel: The current voltage curves of kainate-triggered response were obtained by current amplitudes at the corresponding membrane potentials at the peak response during the kainate application (2). The reversal potential was at 0 mV.

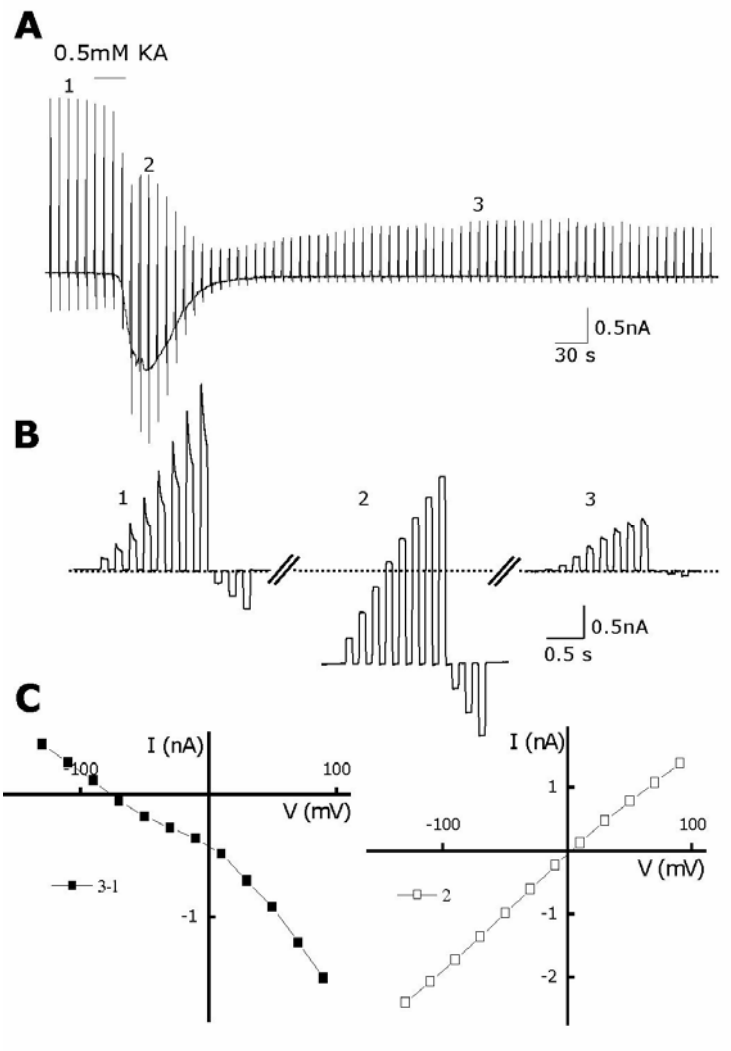


Fig.3.13. Another different profile of kainate evoked response current in nestin-GFP-expressing cells in the amygdala

A: Similar as described in the legend to Fig.3.12, kainate (0.5 mM, 30 sec) was applied while recording membrane currents from an other GFP- expressing cell, which display differently kainate-triggered response profile over the time (n=10 out of 21). B: Three series of voltage steps from the recording shown in (A) before (1), at the peak of the kainate response (2) and long term blockage of the resting K^+ conductance (3), are shown with higher time resolution and were used to construct a current voltage curve as shown C: Left panel: The current voltage curves of long term blockage of the resting K^+ conductance were obtained by subtracting current amplitudes at the corresponding membrane potentials before and about 12 mins after the kainate application (3-1). The reversal potential was close to -80 mV. C: Right panel: The current voltage curves of kainate-triggered response were obtained by current amplitudes at the corresponding membrane potentials at the peak response during the kainate application (2). The reversal potential was at 0 mV.

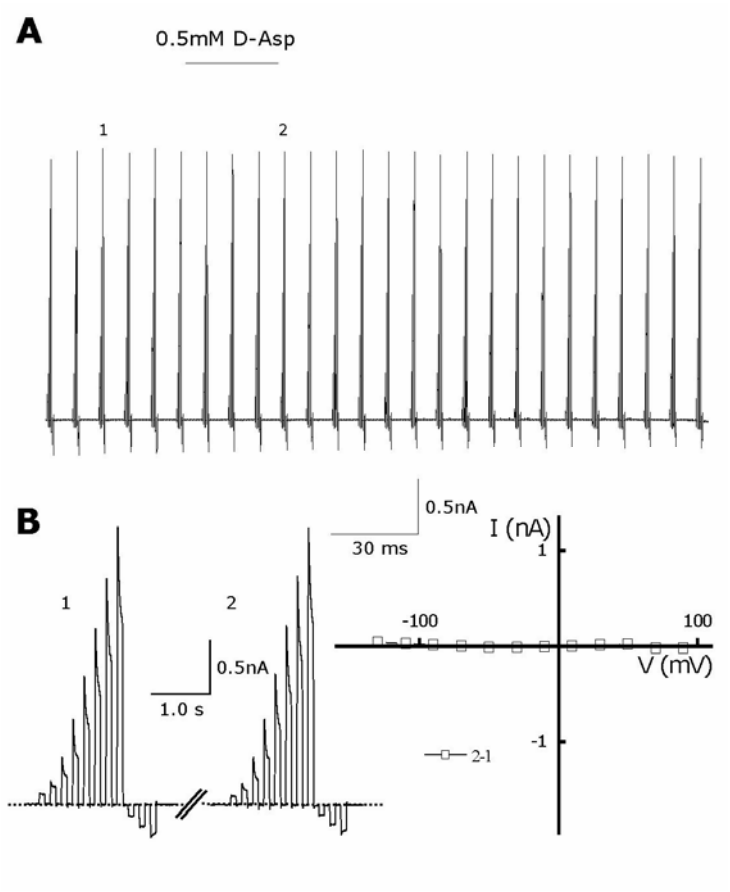


Fig.3.14. Lacking the glutamate transporter currents expression in the nestin-GFP-expressing cells in the amygdala

A: Similar as described in the legend to Fig.3.12, there is no current response to D-Aspartate application (0.5 mM, 30 sec) was analyzed from a ´complex´ nestin-GFP- expressing cell (n=10 out of 10). B: Left panel, two series of voltage steps from the recording shown in (A) before (1) and during the D-Aspartate application (2), construct a current voltage curve as shown in the right panel, there is no current was recorded by the D-Aspartate application.

therefore studied the functional expression of AMPA/kainate glutamate receptors and glutamate transporters in nestin-GFP-expressing cells in the amygdala. Bath application of kainate (0.5mM, 30sec) triggered an inward current in all measured cells (n=29 out of 29) while clamping the membrane at –70mV (Fig.3.12, 3.13). To determine the reversal potential of the kainate response, the membrane was clamped to a series of de- and hyperpolarizing potential ranging from –130 to 90 mV from a holding potential of –70 mV with 20 mV increments. These series of voltage jumps was repetitively applied and allowed us to study the conductance changes over time and construct I/V curves in 6 s intervals.

Kainate evoked a complex response, which was composed of three different components (Fig.3.12). Within few seconds after application, the resting (K^+) conductance decreased and the reversal potential of this blocked conductance was at 0 mV (Fig.3.12, C right panel). Both the inward and the outward K^+ conductance were affected. Subsequently a conductance increase developed which had a reversal potential of 0 mV indicating that a cationic current was activated. This conductance increase returned to baseline within about 4 min after washout of kainate. With a delay of 3 to 4min after washout of kainate, an outward rectifying current developed with a reversal potential of -70 mV (Fig.3.12, C left panel). This outward K^+ conductance returned to baseline after about 5 min. The resting K^+ conductance, which was reduced in amplitude at the beginning of the response, remained reduce, even after 20 min recording time. In conclusion, kainate triggers a rapid and long-lasting blockade of the resting K^+ conductance, a transient cationic current and, with a significant delay, a transient increase of an outwardly directed K^+ conductance. There was some heterogeneity with respect to the late developing K^+ outward conductance: it was missing in 9 out of 29 cells (Fig.3.13).

We also studied for the presence of glutamate transporter currents by applying D-aspartate, which are a substrate for the glutamate transporter and not a ligand for glutamate receptors. No currents were recorded after D-aspartate application (n= 20; Fig.3.14). Thus, we concluded that the nestin-GFP-expressing cells in the amygdala striatum express functional glutamate receptors of the AMPA/Kainate subtype, but no glutamate transporter expression.

3.5. Membrane current features of nestin-GFP-expressing cells in alveus CA1 and corpus callosum

3.5.1. Nestin-GFP-expressing also be found in corpus callosum, CA1 and alveus

Using the nestin-GFP expressing mice, we can found high numbers of GFP-positive cells all over the brain. In addition to the SGZ of hippocampus and SVZ of lateral ventricles, in CA1 of hippocampus, we also found another type nestin-GFP-expressing cell, which was characteristic by their intricate processes in all directions, giving the cells a spongiform- or dandelion-like appearance. Nestin-GFP-expressing cells also present in the corpus

callosum and below the corpus callosum, in a small band of tissue referred to as alveus. In both of these areas cells tended to show the morphology of migrating cells with two short processes on opposite sides of the cell soma oriented parallel to the fiber tracts. Between CA1 and alveus a small number of bipolar nestin-GFP-positive cells with large processes were found, which were usually oriented perpendicular to the corpus callosum and CA1.

3.5.2. Astrocytic properties in nestin-GFP-expressing cells in alveus and CA1

Nestin-GFP expressing spongiform cells also displayed a current pattern characteristic for hippocampal astrocytes. In the CA1 region of the hippocampus we selected for GFP-positive cells which had the typical morphology of spongiform cells: In the fluorescence image, these cells were characterized by small somata and multiple processes and were surrounded by a diffuse fluorescence. After recording, the cells were filled with Alexa Fluor 594, which yielded an even better image of their morphology (Fig.3.15, A-D). After establishing the whole cell-recording configuration, cells were clamped at a holding potential of –70 mV. We selected for cells with a stable membrane potential, which was between –50 and –75mV (N=86). The input resistance was 89.8 ± 39 mΩ in nestin-GFP-expressing protoplasmic astrocytes. In the voltage clamp mode, membrane currents were activated by clamping the membrane from the holding potential to hyper- and depolarizing potentials ranging from –140 to 20 mV (50 ms, 10 mV increment). In the majority of cells (38 out of 40) the currents did not show any time dependence at any voltage. The resulting current voltage curve was linear with a reversal potential at –74 mV (N=40) thus close to the K^+ equilibrium potential (-90mV). This current pattern is characteristic for the majority of astrocytes in the hippocampus (Steinhauser et al., 1994). The remaining two cells displayed an additional delayed activating, inactivating current component that was superimposed on the large passive currents.

To test for the degree of coupling, a cell was dialyzed with the gap junction-permeable fluorescent dye Alexa 594. For these experiments we only labeled one cell per slice. In only 4 out of 40 slices, the dye also labelled one to eight neighboring cells (mean 4) indicating gap junctional coupling.

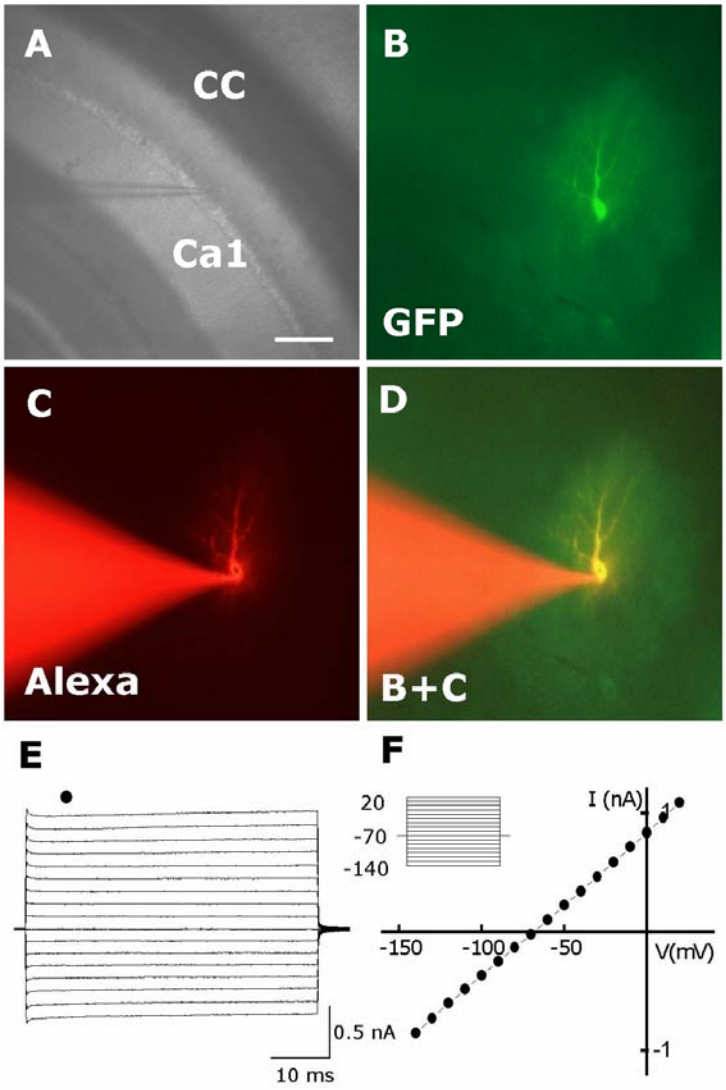


Fig.3.15. Morphology and current pattern of nestin-GFP-expressing cells in the CA1

A: Bright-field images of a hippocampus slice showing the position of the patch pipette during recording. B: Fluorescence image of GFP- expressing cells (excitation at 490 nm and emission at 535 nm). The cell marked by arrow was recorded from and dialyzed with Alexa Fluro 594 via the pipette solution. C shows the Alexa Fluro 594 staining (excitation at 590 nm and emission at 645 nm). D: The merged image verifies that the GFP and Alexa Fluro 594 labels correspond. E: Membrane currents from the cell shown in A-D were evoked by 50 msec voltage steps ranging from –140 mV to +20 mV from holding potential of –70 mV (the recording protocol see the inset in F). F: Right panel: The linear current to voltage curve was obtained from E; current amplitudes at the beginning black circle were plotted against the holding potential. Scale bar in A denotes 200 μm in A, 25 μm in B, C, and D. Scale bars in E represent 10 ms and 0.5nA.

3.5.3. Properties of small nestin-GFP-expressing bipolar cells in alveus and corpus callosum

Smaller nestin-GFP-expressing cells were located in the alveus/ corpus callosum. These cells were characterized by their longish morphology and by the absence of elongated processes (Fig. 3.16,A-D). Electrophysiological recordings for nestin-GFP-expressing cells in the corpus callosum, we selected for GFP-positive cells with an oval soma in the orientation of the fiber tract. In most cells, small processes could be recognized which were also oriented along the fiber

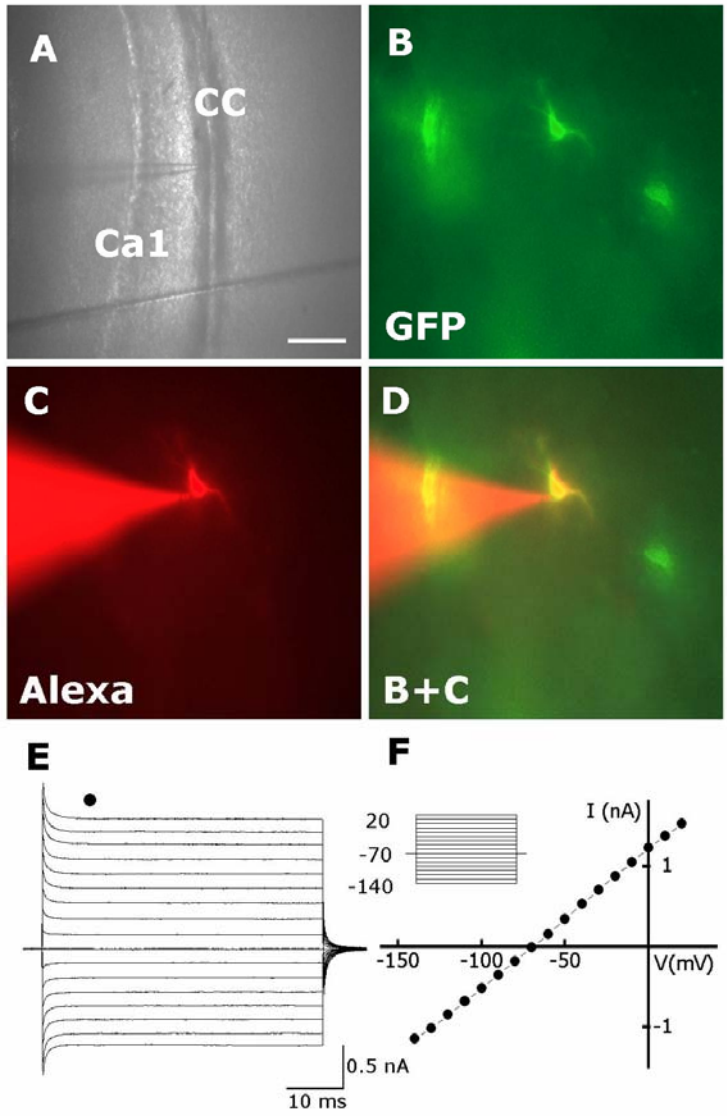


Fig.3.16. Morphology and current pattern of nestin-GFP-expressing cells in the alveus/ corpus callosum

A: Bright-field images of a hippocampus slice showing the position of the patch pipette during recording. B: Fluorescence image of GFP- expressing cells (excitation at 490 nm and emission at 535 nm). The cell marked by arrow was recorded from and dialyzed with Alexa Fluro 594 via the pipette solution. C shows the Alexa Fluro 594 staining (excitation at 590 nm and emission at 645 nm). D: The merged image verifies that the GFP and Alexa Fluro 594 labels correspond. E: Membrane currents from the cell shown in A-D were evoked by 50 msec voltage steps ranging from –140 mV to +20 mV from holding potential of –70 mV (the recording protocol see the inset in F). F: Right panel: The linear current to voltage curve was obtained from E; current amplitudes at the beginning black circle were plotted against the holding potential. Scale bar in A denotes 200 μm in A, 25 μm in B, C, and D. Scale bars in E represent 10 ms and 0.5nA.

tract. The majority of these cells (31 of 34) displayed currents similar to those described for the nestin-GFP-expressing astrocytes. Three remainder cells displayed an additional delayed activating current component. The input resistance of these cells was 99.5 ± 59 mΩ. We also observed dye coupling in three out of 34 experiments. These cells were dye-coupled to one or two cells.

3.5.4. Nestin-GFP-expressing cells with large bipolar processes oriented perpendicular to the corpus callosum/ pyramidal cell layer are characterized by passive membrane currents

Again, we studied the membrane currents of these large bipolar cells with the patch-clamp with the same paradigm as described above for the nestin-GFP-expressing protoplasmic cells in CA1 (Fig.3.17, A-D). All bipolar cells (n=12) displayed passive currents similar to those described above. The input resistance of bipolar cells was higher than that obtained for the nestin-GFP protoplasmic astrocytes, namely 143.1 ± 82.7 mΩ. We also observed dye coupling in a third of the bipolar cells (N=12), which were coupled to one to 12 cells (mean 4).

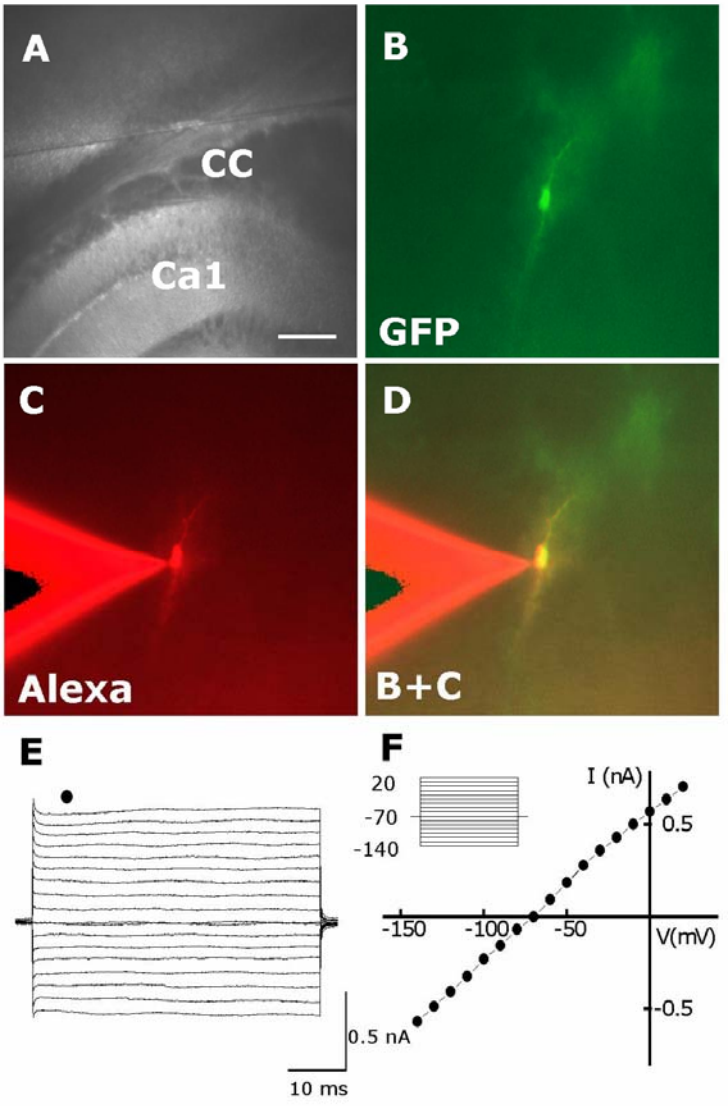


Fig.3.17. Morphology and current pattern of large bipolar processes nestin-GFP-expressing cells.
A: Bright-field images of a hippocampus slice showing the position of the patch pipette during recording. B: Fluorescence image of GFP- expressing cells (excitation at 490 nm and emission at 535 nm). The cell marked by arrow was recorded from and dialyzed with Alexa Fluro 594 via the pipette solution. C shows the Alexa Fluro 594 staining (excitation at 590 nm and emission at 645 nm). D: The merged image verifies that the GFP and Alexa Fluro 594 labels correspond. E: Membrane currents from the cell shown in A-D were evoked by 50 msec voltage steps ranging from –140 mV to +20 mV from holding potential of –70 mV (the recording protocol see the inset in F). F: Right panel: The linear current to voltage curve was obtained from E; current amplitudes at the beginning black circle were plotted against the holding potential. Scale bar in A denotes 200 μm in A, 25 μm in B, C, and D. Scale bars in E represent 10 ms and 0.5nA.

3.6. Membrane current features and functional expression of AMPA/Kainate receptors by nestin-expressing precursor cells in the adult striatum after stroke

3.6.1. Nestin-GFP-expressing cells acquire the 'complex' physiological phenotype after stroke

To analyze the physiological properties of astrocytes after an ischemic insult, we isolated striatal brain slices from nestin-GFP transgenic mice at different time points after MCAo/ reperfusion. After establishing the whole cell-recording configuration (Hamill et al., 1981) the membrane currents were studied by clamping the membrane at different potentials (Fig.3.18, A-D). We selected for cells with a stable membrane potential, which was between -50 and -85mV (n=111). The membrane was clamped from the holding potential of -70 mV to hyper- and depolarizing potentials ranging from -180 to +80 mV (50 ms, 10 mV increment). Based on the membrane currents, we could distinguish two populations of nestin-GFP-expressing cells. One population was characterized by outwardly rectifying currents activated by depolarization and inward rectifying, inactivation currents induced with hyperpolarization (Fig.3.18, E). This current pattern is characteristic for ´complex astrocytes´, a subtype of astrocytes first described in the hippocampus(Jabs et al., 1994; D'Ambrosio et al., 1998) and we will therefore refer to these cells as ´complex cells´. The current profile of the second subpopulation of nestin-GFP-expressing cells was characterized by time and voltage-independent currents (Fig.3.18, F). The resulting current/ voltage curve was linear with a reversal potential at -74 mV close to the K^+ equilibrium potential. This current profile is characteristic for the majority of astrocytes in the hippocampus (Steinhauser et al., 1992; D'Ambrosio et al., 1998) and we refer to these cells as "passive cells". We studied the electrophysiological properties of nestin-GFP-expressing cells at different time points after MCAo/ reperfusion. In control animals, the population of complex versus passive cells was about similar (5 complex versus 7 passive cells). A similar result was obtained in sham-operated animals. 48 h after MCAo/ reperfusion, the relative number of complex cells increased (to 78.6%). While 4 and 7 after MCAo/ reperfusion, only complex cells were found among the nestin-GFP population. After 4 and 8 weeks, we again found passive cells, but less as compared to unlesioned controls (10% and 25% of passive cells after 4 and 8 weeks, respectively, Fig. 3.18,G).

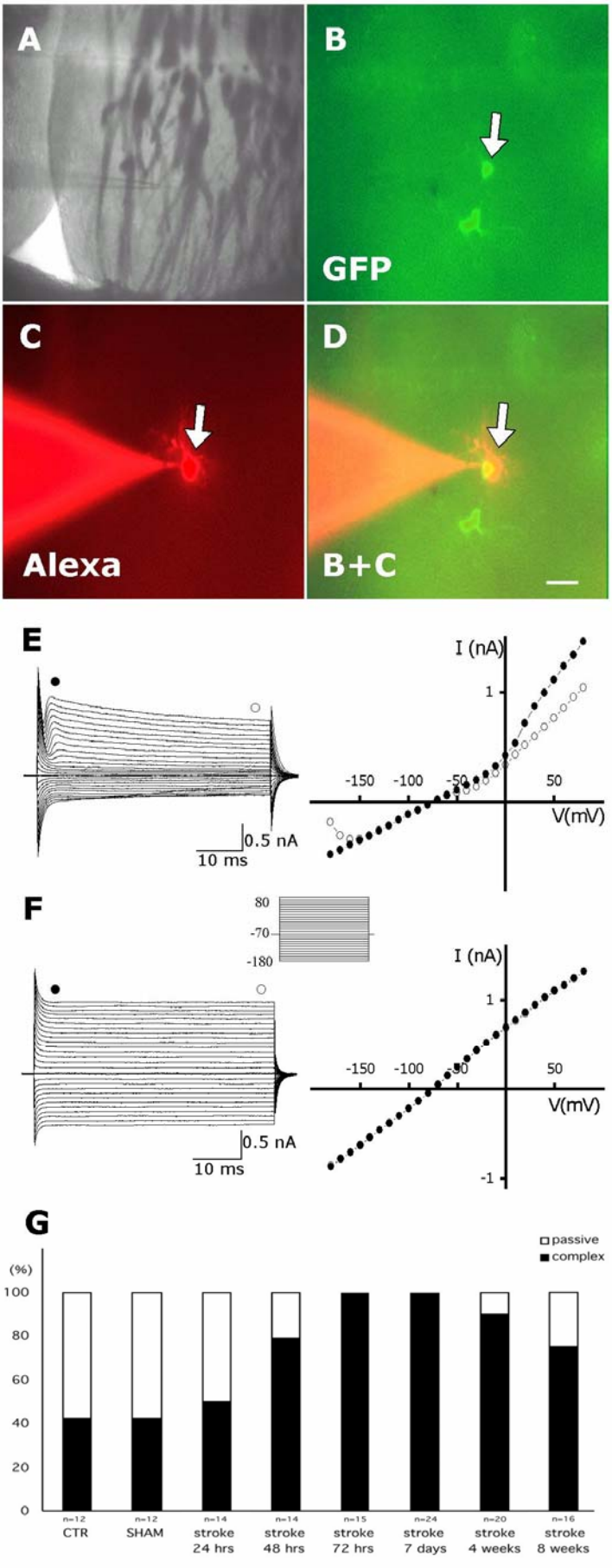

A
B
GFP
C
Alexa
D
B+C
E
I (nA)
1
-150
-100
-50
50
V(mV)
0.5 nA
10 ms
F
80
-70
-180
I (nA)
1
-150
-100
-50
50
V(mV)
-1
0.5 nA
10 ms
G
passive
complex
(%)
100
80
60
40
20
0
n=12
CTR
n=12
SHAM
n=14
stroke
24 hrs
n=14
stroke
48 hrs
n=15
stroke
72 hrs
n=24
stroke
7 days
n=20
stroke
4 weeks
n=16
stroke
8 weeks

Fig.3.18. Morphology and current pattern of nestin-GFP+ cells in the striatum of adult nestin promoter-GFP transgenic mice after MCAo/ reperfusion.

A. Bright-field image of a striatal slice showing the position of the patch pipette during recording. B. Fluorescence image of GFP+ cells (excitation at 490 nm and emission at 535 nm). The cell marked by arrow was used for recording and dialyzed with Alexa Fluor 594 via the pipette solution. C. Shows the Alexa Fluor 594 staining (excitation at 590 nm and emission at 645 nm). D. The merged image confirms that GFP and Alexa Fluor 594 label the same cell. E. Left panel: Membrane currents from the cell shown in A-D were evoked by 50 msec voltage steps ranging from –210 mV to +80 mV from a holding potential of –70 mV (recording protocol see the insert in E). Right panel: The outwardly rectifying and inward rectifying current to voltage curve was obtained from recordings shown on the left panel; current amplitudes at the beginning (black circle) and at the end of the voltage pulse (open circle) were plotted against the holding potential. F. Analysis of another GFP-positive cell, which was characterized by a linear current voltage curve. G. Current pattern description of nestin-GFP+ cells at different time points after MCAo/ reperfusion. The results for the 72 hours and the 7 days time point each were obtained from 2 animals (analyzed between 72 hours and 96 hours or between 7 and 8 days after MCAo/ reperfusion, respectively). Scale bar denotes 120 μm in A, 15 μm in B, C, and D.

3.6.2. The ´complex cells´ express AMPA/kainate glutamate receptors, but lack glutamate transporter currents

´Complex astrocytes´ in the hippocampus are characterized by the expression of AMPA-type glutamate receptors and by the lack of glutamate transporters (Matthias et al., 2003). We therefore tested for the presence of AMPA receptors and transporter activity in the Nestin-GFP-expressing cells in striatum after MCAo/reperfusion. Bath application of kainate as a ligand for AMPA receptors (0.5mM, 30sec) triggered an inward current in all cells studied (n=21) while clamping the membrane at –70mV (Fig.3.19; Fig3.20). To determine the reversal potential of the kainate response, the membrane was clamped to a series of de- and hyper-polarizing potential ranging from –130 to 90 mV from a holding potential of –70 mV with 20 mV increments. These series of voltage jumps was repetitively applied and allowed us to study the conductance changes over time and construct I/V curves in 6 s intervals. By subtracting I/V curves from each other obtained at different time points, we could isolate current components, which developed over time.

Kainate evoked a complex response, which was composed of three different components. (n=10 out of 21 cells)(Fig.3.19). Within few seconds after application, the resting (K^+) conductance decreased and the reversal potential of this blocked conductance was at 0 mV. Both the inward and the outward K^+ conductance were affected. Subsequently a conductance increase developed which had a reversal potential of 0 mV indicating that a cationic current was activated (Fig.3.19 C, right panel). This conductance increase returned to baseline within about 4 min after washout of kainate. With a delay of 3 to 4min after washout of kainate, an outward rectifying current developed with a reversal potential of -70 mV (Fig.3.19, C, left panel). This outward K^+ conductance returned to baseline after about 5 min. The resting K^+ conductance, which was reduced in amplitude at the beginning of the response, remained reduce, even after 20 min recording time. In conclusion, kainate triggers a rapid and long-lasting blockade of the resting K^+ conductance, a transient cationic current and, with a significant delay, a transient increase of an outwardly directed K^+ conductance. There was some heterogeneity with respect to the late developing K^+ outward conductance: it was recorded in only about half of the cells (n=11 out of 21 cells)(Fig.3.20).

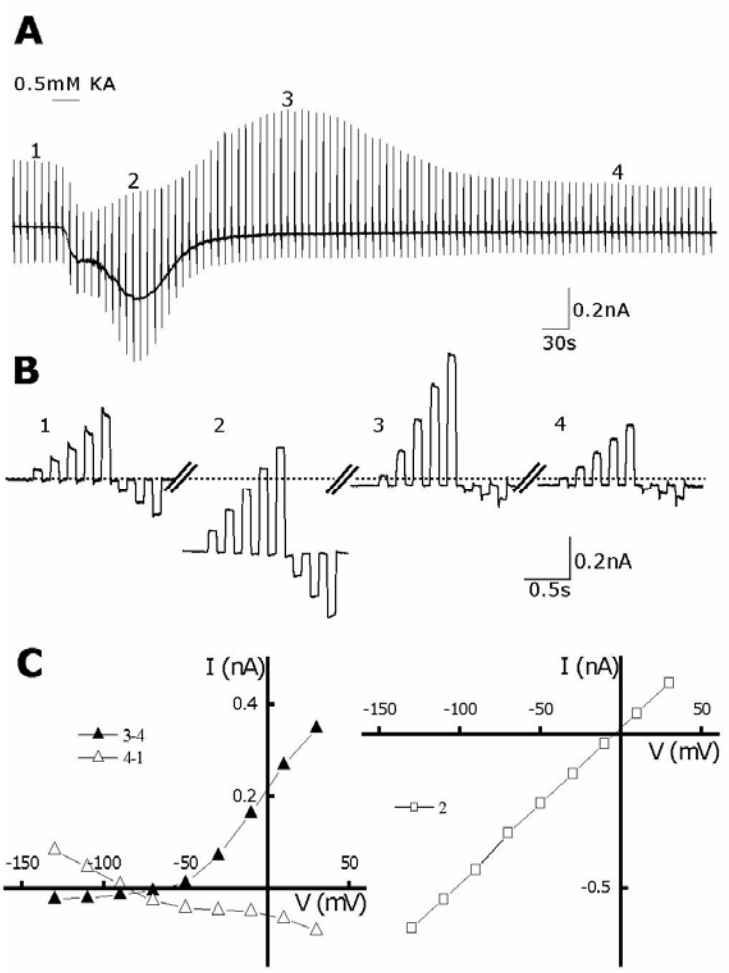


Fig.3.19. Functional expression of AMPA/kainate glutamate receptors in "complex" nestin-GFP+ cell with three current components

A. During recording of membrane currents from a nestin-GFP+ cell, kainate (0.5 mM, 30 sec) was applied as indicated. The membrane was clamped from a holding potential of -70 mV to a series of de- and hyperpolarizing potentials ranging between -130 mV to +30 mV with 20 mV increments. At this temporal resolution, the single voltage steps cannot be resolved. B. Four series of voltage steps from the recording shown in (A) before (1), at the peak of the kainate response (2), the development of an outward rectifying current (3) and of long term blockage of the resting K^+ conductance (4), are shown with higher time resolution and were used to construct a current voltage curve as shown in (C). C. Left panel: The current voltage curves of long term blockage of the resting K^+ conductance were obtained by subtracting current amplitudes at the corresponding membrane potentials before and about 12 min after kainate application (4-1). The reversal potential was close to -80 mV. The current voltage curves of the outward rectifying current were obtained by subtracting current amplitudes at the corresponding membrane potentials at the peak of the outward rectifying current and about 12 min after kainate application (3-4). The reversal potential was close to -70 mV. Right panel: The current voltage curves at the peak of the inward current (at –70 mV) of the kainate-triggered response was obtained by current amplitudes at the corresponding membrane potentials at the peak response during the kainate application (2). The reversal potential was at 0 mV.

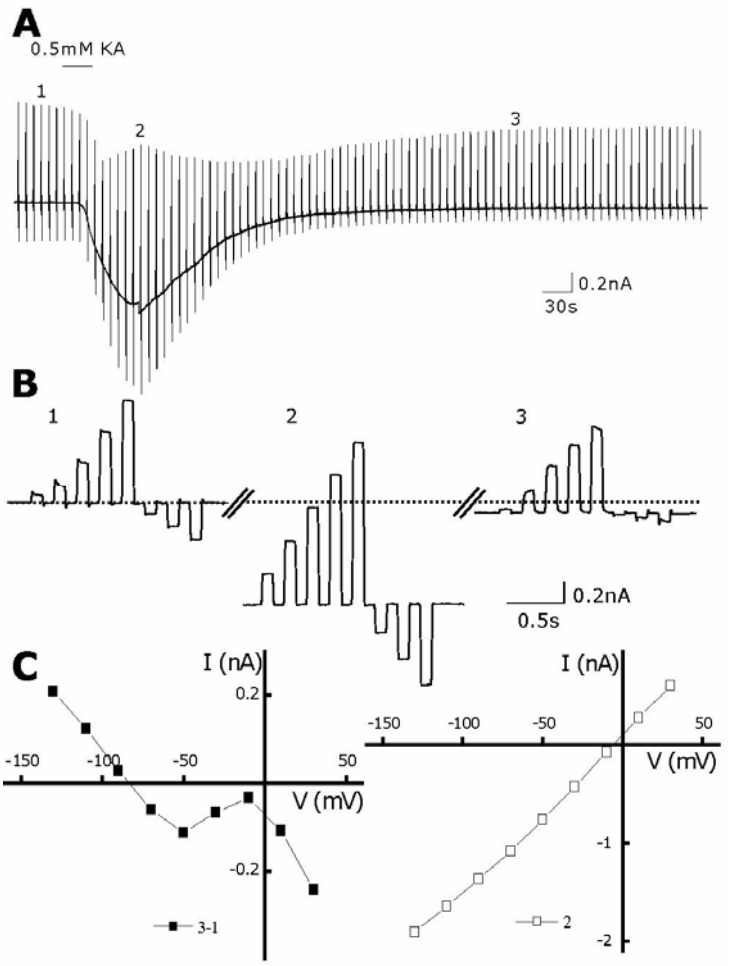


Fig.3.20. Functional expression of AMPA/kainate glutamate receptors in "complex" nestin-GFP+ cell with two current components

A. As described in the legend to Fig.3.19, kainate (0.5 mM, 30 sec) was applied while recording membrane currents from another nestin-GFP+ cell, which displayed a somewhat different kainate-triggered response profile over time (n=10 out of 21 cells analyzed). B. Three series of voltage steps from the recording shown in (A) before (1), at the peak of the kainate response (2) and at the time of long term blockage of the resting K+ conductance a later time point (3), are shown with higher temporal resolution and were used to construct a current voltage curve as shown in (C). C. Left panel: The current voltage curves of long term blockage of the resting K+ conductance were obtained by subtracting current amplitudes at the corresponding membrane potentials before and about 12 mins after kainate application (3-1). The reversal potential was close to -80 mV. Right panel: The current voltage curves of the kainate-triggered response at the peak of the inward current (at –70 mV) wasere obtained by plotting current amplitudes and at the corresponding membrane potentials at the peak response during the kainate application (2). The reversal potential was at 0 mV.

We also studied for the presence of glutamate transporter currents by applying D-aspartate, which are a substrate for the glutamate transporter and not a ligand for glutamate receptors. No currents were recorded after D-aspartate application (Fig.3.21) (n= 21). Thus, we concluded that the ´complex´ nestin-GFP-expressing cells in striatum express functional glutamate receptors of the AMPA/Kainate subtype, but no glutamate transporter expression and that this cell population dominates after MCAo/reperfusion.

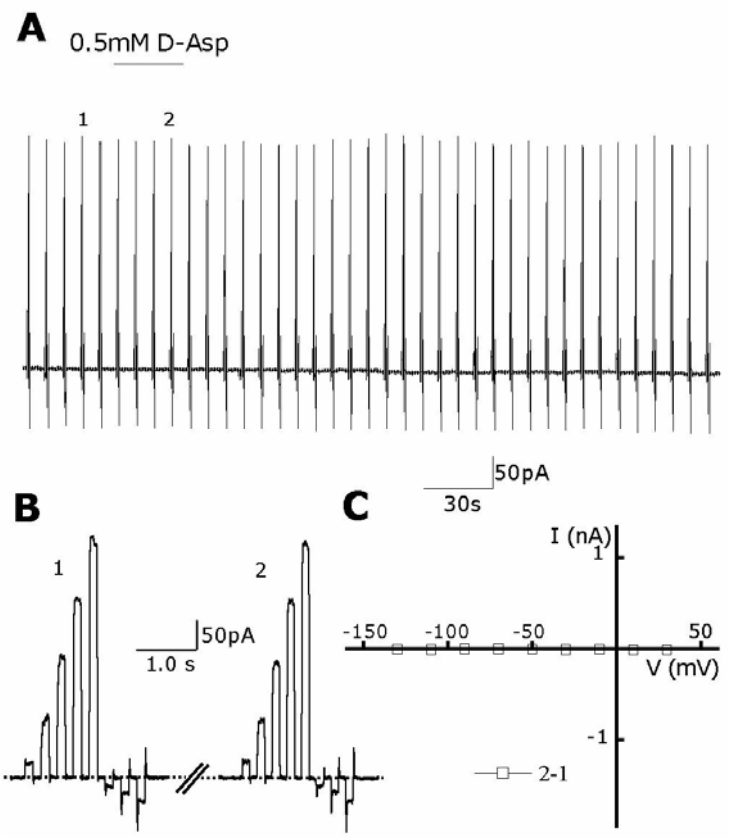


Fig.3.21. "Complex"nestin-GFP+ cells lack glutamate transporter currents.
A. There was no current response to D-aspartate application (0.5 mM, 30 sec) in any complex nestin-GFP+ cell analyzed (n=10 out of 10). B. Two series of voltage steps from the recording shown in (A) before (1) and during D-aspartate application (2), were used to construct a current voltage curve as shown in (C). C. No current was recorded following D-aspartate application.

4. Discussion

4.1. One subpopulation of nestin-expressing progenitor cells share astrocytic features in the adult murine hippocampus

Nestin is a class IV intermediate filament protein (Hockfield and McKay, 1985; Frederiksen and McKay, 1988). The nestin gene contains an enhancer in its second intron that only expression to the CNS precursor cells (Zimmerman et al., 1994). In vitro study, Lendahl et al. found that nestin is expressed only in precursor cells (Lendahl et al., 1990). Other evidences further supported that nestin expression is also restricts in precursor cells in the adult DG (Palmer et al., 2000; Blumcke et al., 2001; Nacher et al., 2001). However, in the adult mouse dentate gyrus, nestin expression is low, so that antibody stainings are cumbersome and show a misleading cross-reaction with a vascular antigen (Palmer et al., 2000). The use of the nestin-GFP reporter mouse allows circumventing this problem (Yamaguchi et al., 2000). The cytoplasmic GFP diffuses through the entire cell and allows visualization of their complete morphology. The use of the neural specific enhancer element of the nestin regulatory region limits GFP expression to neuroepithelial cells (Yamaguchi et al., 2000). When the progenitor cells mature, the nestin promoter will shut down and GFP fluorescence and nestin immunoreactivity will disappear. Using the same nestin-GFP transgenic mouse line, neural progenitor cells have been successfully isolated, which display the stem cell properties (Kawaguchi et al., 2001; Sawamoto et al., 2001b; Sawamoto et al., 2001a).

Astrocytes are the stem cells of the adult SGZ (Alvarez-Buylla et al., 2001; Seri et al., 2001). Usage of the nestin-GFP transgenic mouse, we identified the two subpopulations of nestin-GFP-expressing cells in adult DG by their morphological criteria. We applied immunohistochemical, ultrastructural, and electrophysiological approaches, demonstrated that type-1 nestin-GFP-expressing cells share features with mature astrocytes: with long process express GFAP, which linking this cell type with the “B cells” as described in (Seri et al., 2001); vascular end feet by electron microscopy study and typical electrophysiological features of astrocytes which was characteristiced by passive, noninactivating currents with a linear current–voltage relationship and a reversal potential close to the K^+ equilibrium potential (Steinhauser et al., 1992; Steinhauser et al., 1994).On the contrast with type-1 nestin-GFP-expressing cells, type-2 cells, which has shorter

process and smaller somata compare with type-1 cells and GFAP negative, electrophysiologically with characteristics of delayed-rectifier K^+ currents resembling the electrophysiological description of a glial precursor cell (Berger et al., 1991). Only a few nestin-GFP-expressing cells showed neuronal membrane properties with voltage-gated sodium currents, but all the cells belonged to the type-2 cells, by which mirror the transition state from nestin-GFP-expressing type-2 progenitor cells toward neuronal development. Detailed discussion of this part by (Filippov et al., 2003).

These findings also raised another idea. Electrophysiologically, cells with voltage-gated channels similar to glial precursor cells or the recently described as ´receptor´ astrocyte in hippocampus (Matthias et al., 2003), and cells with sodium currents like in neurons, corresponded to the nonastrocytic type-2 cells. In the following study, we have focused on the type-2 cells, since they are the first candidates to acquire neuronal properties based on our current hypothesis of neuronal development in the adult dentate gyrus (Kempermann et al., 2004). Whether or not the putatively precursor cells in dentate gyrus are integrated into the brain circuitry? And how?

4.2. Earliest synaptic input during adult hippocampal neurogenesis is GABAergic

It is now well established that stem cells in the adult hippocampus generate neurons, which integrate into the existing neuronal network. These newly generated granule cells are produced in the dentate gyrus and project to the CA3 layer where they form synapses (Markakis and Gage, 1999). Using a green fluorescent protein (GFP)-expressing retrovirus as a marker of dividing cells, (van Praag et al., 2002) studied newly generated granule cells in acutely isolated slices and demonstrated that these cells receive synaptic input from the perforant path. Wang et al. (Wang et al., 2000) and Schmidt-Hieber et al. (Schmidt-Hieber et al., 2004) demonstrated that the immature granule cells have a reduced threshold for long-term potentiation. When adult neural stem cells were isolated and cocultured with primary neurons from hippocampus, they could integrate into existing networks and exhibit both GABAergic and glutamatergic postsynaptic responses (Toda et al., 2000; Song et al., 2002). Similarly, embryonic stem cells, when grafted into brain slices or intact brain, received both GABAergic and glutamatergic input (Englund et al., 2002; Benninger et al., 2003). During development, however, the establishment of synaptic connections

follows a defined sequential pattern: First, neurons receive GABAergic input, which is excitatory due to a depolarizing action of GABA at this stage. Subsequently, glutamatergic input is established (Ben-Ari, 2002). A similar developmental sequence was also reported for granule cells during development: postsynaptic currents are first dominated by GABAergic neurotransmission, and the contribution of glutamatergic neurotransmission increases with neuronal maturation (Liu et al., 1996). The plexus of inhibitory GABAergic axons is already well developed at a stage when the granule cells are still being formed (Lubke et al., 1998). In the present study we aimed at identifying such sequential establishment of synaptic input during adult neurogenesis.

We have previously shown that nestin-GFP expressing precursor cells in the adult subgranular zone (SGZ) of hippocampal dentate gyrus could be divided into two distinct subpopulations based on morphological criteria (Filippov et al., 2003). Type-1 cells expressed glial fibrillary acidic protein (GFAP), and extension of a long radial-glia-like process spanning the entire granule cell layer. They showed low proliferative activity, whereas type-2 cells had shorter horizontal or no processes, lacked expression of GFAP and were highly proliferative. Precursor cells were identified using a transgenic animal in which the nestin promoter drives expression of the GFP (Yamaguchi et al., 2000). The nestin-GFP-positive cell population was also distinct by its membrane current pattern: we found (1) cells with passive membrane properties similar to the classical astrocytes, (2) cells with voltage-gated channels similar to glial precursor cells or the newly described ´receptor´ astrocyte (Matthias et al., 2003), and (3) cells with sodium currents like in neurons. In the present study, we have focused on the type-2 cells since they are the first candidates to acquire neuronal properties based on our current hypothesis of neuronal development in the adult dentate gyrus (Kempermann et al., 2004). By stimulating the perforant path, we tested for the first synaptic input into this cell population.

4.2.1. The type-2 nestin-positive population contains the earliest form of neurons during maturation

As we have discussed above, the nestin-GFP reporter mouse provided the unique tool to study adult neurogenesis (Yamaguchi et al., 2000). Especially for the electrophysiology study of the putative progenitor cells feature based on their morphology criteria in situ. Under the neural specific enhancer element of the nestin regulatory region restricts GFP expression only in putative progenitor cells. When the progenitor cells mature, the nestin

promoter will shut down and GFP fluorescence and nestin immunoreactivity will disappear. While we have no information on the decay of the nestin protein level, extrapolating results from the mouse LA-9 cell line, the half life of GFP is about 26 hours (Corish and Tyler-Smith, 1999) which is in line with our earlier studies where we have seen marker overlaps not much exceeding two or three days (Brandt et al., 2003; Kronenberg et al., 2003; Kempermann et al., 2004; Steiner et al., 2004). Histochemically, we have found a very small number of nestin-GFP-positive cells that expressed the post-mitotic neuronal markers calretinin or NeuN. There is also an overlap with the expression of transcription factor Prox1 (Kronenberg et al., 2003), which is characteristic of granule cells. It is not yet known at which stage of development Prox1 is switched on, but all postmitotic cells express Prox1 during adult hippocampal neurogenesis. We therefore assume that our small populations of nestin-positive cells, which can generate action potentials, represent the earliest form of neurons during neurogenesis.

4.2.2. Adult-generated neurons first receive GABAergic input

In late embryonic, early postnatal development, the first functional synapses are GABAergic. This seems to be a universal rule found in different types of species and brain regions and glutamatergic input develops subsequently (Ben-Ari, 2001, 2002). At this developmental stage, GABA acts as an excitatory transmitter due to an increased Cl- level and the resulting depolarizing GABA action. GABA-mediated synaptic activity can trigger action potentials and increases in cytoplasmic Ca^{2+} due to the opening of voltage gated Ca^{2+} channels. This early excitatory input generates a network activity dominated by giant depolarizing potentials (Ben-Ari et al., 1989). Neuronal activity early in development is an important feature controlling maturation of neurons and synapse formation (Katz and Shatz, 1996; Penn and Shatz, 1999).

In a study on newborn hippocampus, Tyzio et al.(Tyzio et al., 1999) described three types of neurons, ´silent neurons´ which are devoid of synaptic input and lack dendrites, ´GABA-only neurons´ that receive GABAergic input and have a only small apical dendrite and ´GABA-and-glutamate neurons´ which receive both GABAergic and glutamatergic input and a more elaborate dendritic arborization. Tyzio et al. imply that this pattern represents a developmental sequence and this is supported by studies from primate neurons in utero (Khazipov et al., 2001). In analogy to this developmental study, we found both ´silent neurons´ and ´GABA-only neurons´ in our population of the type-2 nestin-positive cells from the young adult hippocampus. In contrast to early development, the adult

´GABA-only neurons´ did not have an apical dendrite. Interestingly, like in our study, the silent neurons in the newborn hippocampus expressed functional GABAA and glutamate receptors. The ´GABA-and-glutamate neurons´ of the adult neurogenesis have been described by Schmidt-Hieber et al. (Schmidt-Hieber et al., 2004) as the population of PSA-NCAM positive granule cells, which express different properties than mature granule cells, but receive glutamatergic input. This corresponds to the next step in adult neurogenesis after the progenitor cell stage examined in the present study. In conclusion, neuronal maturation in adult neurogenesis also involves three stages and thus mimics neuronal maturation during development.

We have previously found that during adult neurogenesis a 5- to 6-fold expansion of precursor cells occurs, which leave the cell cycle and show signs of early neuronal maturation. If we assume that the nestin-promoter has shut down at the latest, when the cells leave the cell-cycle, our present data indicate that within a short period neurons matures to a stage where they receive GABAergic input from the perforant path. At that stage, they have not yet developed an extensive dendritic tree nor do they project axons. GABAergic input has been demonstrated to stimulate neuronal development in the hippocampus (Davies et al., 1998), but also other brain structures such as the superior colliculus (Meier et al., 2003). The GABA-mediated early network oscillations are necessary for the induction of functional glutamatergic synapses during early development (Hanse et al., 1997). This could also apply for the maturation of adult immature neurons. The final maturation of new granule cells seems to take weeks (van Praag et al., 2002; Jessberger and Kempermann, 2003; Jonas et al., 2004). After the cells have become postmitotic, they extend dendrites and form axonal projections to CA3. The present finding is consistent with the idea of an early and swift maturation that allows selection of the new neurons, whereas full maturation takes more time.

4.3. Functional relevance of ecto-nucleotidase activity and nucleotide receptors in adult neurogenesis

In the dentate gyrus of hippocampus, radial glia-like cells (residual radial glia) are considered to function as progenitors (Kosaka and Hama, 1986; Schmidt-Kastner and Szymas, 1990) and to maintain their neurogenic potential into adulthood. The GFAP and nestin-expressing residual radial glia proliferates and becomes transformed into neurons via transit amplifying cells (Kaplan and Bell, 1984; Stanfield and Trice, 1988; Seri et al.,

2001; Brown et al., 2003b; Steiner et al., 2004). Cells in the transition state lose the undifferentiated state markers, such as GFAP, nestin, vimentin and start to express the neuronal differentiation markers, such as doublecortin [DCX], βIII-tubulin, Prox-1, calretinin, calbindin, NeuN and migrate into the granule cell layer (Brandt et al., 2003; Brown et al., 2003b; Kronenberg et al., 2003; Mignone et al., 2004; Steiner et al., 2004).

Alvarez-Buylla and colleagues found that GFAP-expressing residual radial glia (B-cells) gives rise to GFAP-negative cells (D-cells) that could function as precursors for generating granule cells in the hippocampus (Seri et al., 2001). We have previously demonstrated that nestin-GFP expressing precursor cells in the adult SGZ of hippocampal dentate gyrus could be divided into two distinct subpopulations based on morphological criteria. Type-1 cells expressed GFAP, and extension of a long radial-glia-like process spanning the entire granule cell layer. They showed low proliferative activity, whereas type-2 cells had shorter horizontal or no processes, which was GFAP-negative and were highly proliferative (Filippov et al., 2003).

NTPDase2 is a member of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family containing a transmembrane-domain at the N- and C-terminus, respectively and a large extracellular loop with the catalytic site. Members of this ecto-nucleotidase family are directly involved in the modulation of ATP receptor-mediated cell communication (Zimmermann, 2000). Zimmermann and colleagues have previously shown that ecto-nucleoside triphosphate diphosphohydrolase 2 (NTPDase2, ecto-ATPase) that hydrolyzes extracellular nucleoside triphosphates such as ATP or UTP to the respective diphosphates serves as a novel marker for type B cells, the stem cells of the SVZ (Braun et al., 2003). NTPDase2 was absent from their presumptive descendants, the highly proliferating type-C cells, migrating neuroblasts (type A cells) and olfactory bulb neurons.

Many evidences supported that nucleotides can control astrocyte or neuronal cell proliferation (Neary and Zhu, 1994; Ryu et al., 2003), migration (Scemes et al., 2003), and differentiation (D'Ambrosi et al., 2001). Extracellular nucleotides can act via ionotropic (P2X, permeable for Na^+, K^+, and Ca^{2+}) or metabotropic (P2Y receptors). These are differentially activated by nucleotide agonists. Whereas P2X receptors are stimulated by ATP, P2Y receptors can be activated by ATP, ADP, UTP, or UDP, depending on subtype (Ralevic and Burnstock, 1998). Various cell types, including cultured astrocytes, express a variety of P2X and P2Y receptors(Fumagalli et al., 2003).Unpublished data from Zimmermann's lab shown that radial glia-like cells in hippocampus express surface-

located ATP-hydrolyzing activity and are immunopositive for NTPDase2 (Fig.4.1). NTPDase2 is expressed in the hippocampus from E17 onwards and its embryonic pattern of expression mirrors dentate migration of neuroblasts. Double-immunolabeling revealed that NTPDase2 is associated with subpopulations of GFAP- and nestin-positive cells as well as by cells positive for doublecortin, which marks the transition of progenitors to a neural phenotype. NTPDase2 is not associated with mature granule cells and S100-positive astrocytes. NTPDase2-positive cells proliferate and postmitotic cells preferentially acquire an NTPDase2-positive phenotype (Fig.4.2).

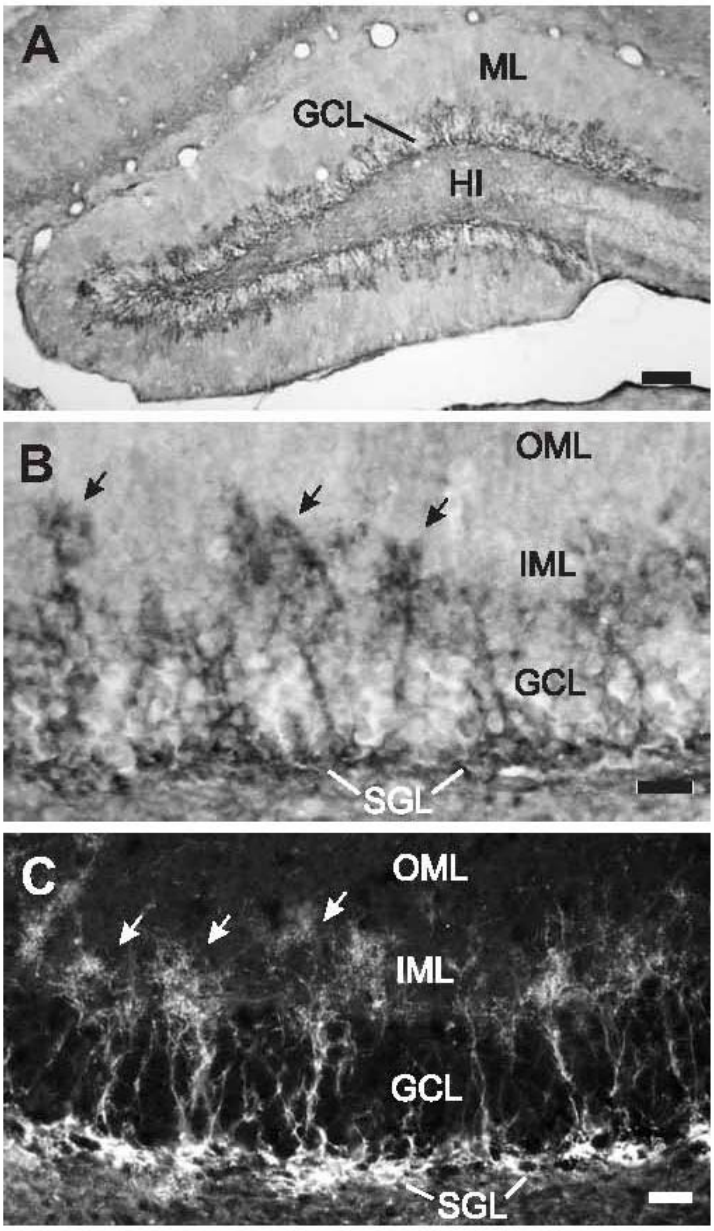


Fig. 4.1. Distribution of ATPase activity and NTPDase2 immunoreactivity in the adult hippocampus. A: Enzyme histochemical staining for ATPase activity depicts intensive staining of radial cells traversing the granule cell layer (coronal section, NTPDase1 knock-out). B: Detail of enzyme histochemical staining for ATPase activity in the dentate gyrus. Note many bush-like arborizations (arrows) at the apical portion of the radial cells (coronal section, NTPDase1 knock-out). C: Immunofluorescence staining for NTPDase2 depicts radial cells traversing the granule cell layer (wild type). Cell bodies reside in the SGL. Trunks traverse the granule cell layer and arborize (arrows) in the inner molecular layer. HI, hilus; GCL, granule cell layer; IML, inner molecular layer; OML, outer molecular layer; SGL, subgranular layer. Scale bar = 100 μm in A; 200 μm in C,D.

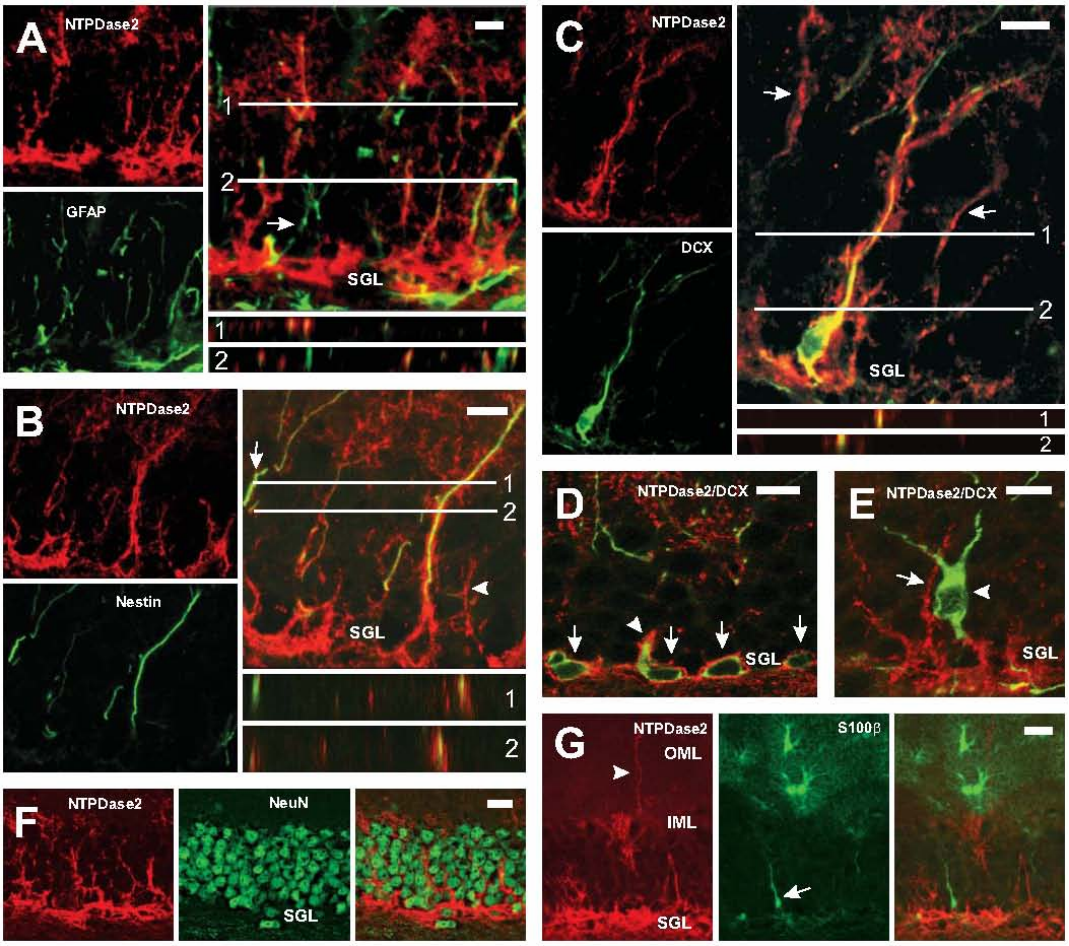


Fig. 4.2. Immunofluorescence analysis of colocalization of NTPDase2 with other cell markers. A-C: Confocal image stacks. Colocalization is visualized at the right panels along the *xz*-axis (bottom image strips). **A:** Double immunofluorescence revealing partial colocalization of NTPDase2 (red, top left) and GFAP (green, bottom left). The arrow in the merged image depicts a radial process positive only for GFAP. **B:** Double immunofluorescence revealing partial colocalization of NTPDase2 (red, top left) and nestin (green, bottom left). The arrow in the merged image depicts a radial process positive only for nestin. **C:** Double immunofluorescence revealing partial colocalization of NTPDase2 (red, top left) and doublecortin (DCX, green, bottom left). The arrows in the merged image depict radial processes positive only for

NTPDase2. **D:** Double immunofluorescence revealing colocalization of NTPDase2 (red) with horizontally oriented DCX-positive cells (arrows) in the SGL. One cell extends a small radial process (arrow). **E:** DCX-positive cell (green, arrow head) within the granule cell layer that is immunonegative for NTPDase2 (red). Arrow: Radial process positive only for NTPDase2. **F:** Double immunofluorescence revealing lack of colocalization of NTPDase2 (red) and NeuN (green). **G:** Double immunofluorescence revealing lack of colocalization of NTPDase2 (red) and S100β (green). The arrowhead marks a radial NTPDase2-positive process reaching into the outer molecular layer, the arrow a radial S100β-positive cell that is negative for NTPDase2. IML, inner molecular layer; OML, outer molecular layer; SGL, subgranular layer. Scale bar = 10 μm in A-E; 20 μm in F,G.

This data strongly supported that the ecto-nucleotidase activity of hippocampal progenitor cells implies that extracellular nucleotides are involved in the control of hippocampal neurogenesis and that NTPDase2 plays a functional role in the extracellular hydrolysis of released nucleotides. This is further supported by our observation that cells expressing GFP under the control of the nestin promotor express functional nucleotide receptors. Interestingly, expression of receptors varied between subtypes. The majority of cells with a radial glia phenotype (type1 cell) expressed ionotropic P2X receptors. We have previously identified that the type-1 cells express passive non-inactivating currents with a linear current voltage relationship and a reversal potential close to the K^+ equilibrium potential (Filippov et al., 2003; Fukuda et al., 2003). Only a minority of cells with a horizontal orientation, which was type 2 cell that previously, was shown to reveal outwardly rectifying properties (Filippov et al., 2003; Fukuda et al., 2003) responded to ATP. This opens the interesting possibility that ATP, released from contacting cells, either the granules cells or the incoming hippocampal commissural-associational fibers that terminate in the inner molecular layer (Brinks et al., 2004), activates the radial progenitor cells via ionotropic P2X receptors. The observed heterogeneity of radial glia regarding the response to ATP further supports the notion of a functional heterogeneity as revealed by our immunostaining analyses.

We observed radial glial cells that were negative for NTPDase2 but positive for either GFAP or nestin. The lineage relation of this subpopulation of cells is not clear but they presumably represent a functionally distinct subpopulation of radial glial cells of the dentate gyrus. Interestingly, a similar heterogeneity regarding membrane current properties was observed for glial cells of the dentate gyrus visualized by expression of EGFP controlled by the human GFAP promotor (Hüttmann et al., 2003). Only a minority of horizontally oriented GFP-positive cells expressed P2X receptors. Also this cell population was found to be functionally heterogeneous with a small subpopulation revealing

functional properties indicative of the presence of voltage gated Na^+ channels (Filippov et al., 2003). Our observation that also DCX-positive cells express NTPDase2 implies that they may equally express functional nucleotide receptors. Our study did not specifically address the expression of P2Y receptors. However, the observation that application of ADP or UTP induced small inward currents would be compatible with a P2Y receptor-mediated indirect effect on current properties due to inhibition of K^+-channels (Barnard et al, 2003).

At present, the impact of nucleotidergic signaling in the neurogenic pathway is not understood. Nucleotides may be involved in the regulation of cell proliferation or also cell differentiation. Nucleotides can induce differentiation of PC12 cells, presumably involving both P2X and P2Y receptors (D'Ambrosi et al., 2001). Nucleotides were shown to exert a synergistic effect on cell proliferation together with growth factors or cytokines (Huang et al., 1989; Lemoli et al., 2004), converging on growth factor-activated signaling pathways (Neary and Zhu, 1994; Lenz et al., 2000). It is possible that the profile of P2 receptor expression changes with progressive cell differentiation (Hogg et al., 2004) and activation of certain P2 receptor subtypes could either favor precursor cell proliferation or differentiation. Additional investigations are required to identify nucleotide receptor subtypes and to elucidate the functional role of NTPDase2 and of nucleotides in hippocampal neurogenesis.

Our results reveal that nestin-GFP-expressing progenitor cells can express P2 receptors in situ, combined with the evidence from Zimmermann's lab, with which supported that NTPDase2 associated with proliferating progenitor cell populations of the dentate gyrus. Therefore, we conclude that the nucleotides may function as extracellular signaling molecules may be involved in the regulation of adult neurogenesis in the dentate gyrus.

4.4. Nestin-GFP-expressing cells display OPC features in amygdala

Unpublished results from Kempermann's lab revealed that nestin-GFP-expressing cell in amygdala expressed the proteogylcan NG2, and also consistently expressed antigenic markers characteristic for stem or progenitor cells (Nestin, Olg2, BLBP) and neurogenic potential cells (Dcx and NeuroD) (data not shown). Electrophysiological properties of nestin-GFP-expressing cells in amygdala displayed outwardly rectifying currents feature and expressed functional AMPA/Kainate subtype glutamate receptors, but no glutamate

transporter expression. In P6 corpus callosum slice, we confirmed that OPCs in corpus callosum (Berger et al., 1991) displayed the similar properties, including electrophysiological and functional AMPA/Kainate subtype glutamate receptor expression, as the nestin-GFP-expressing cells in amygdala (data not shown). We further showed that a proportion of nestin-GFP expressing amygdaloid cells exhibited not only antigenic neuronal but also electrophysiological features typical for neurons and received neuronal GABAergic input. Previously study has demonstrated that OPCs in hippocampus are a direct target of interneuronal collaterals (Lin and Bergles, 2004). At least a subpopulation NG2 positive cells in cortical, instead of white matter, depolarization can elicit immature action potentials-like spikes and expressed AMPA receptors as well (Chmielnicki et al., 2004). Although we never found that nestin-GFP expressing amygdaloid cells coexpressed NeuN, but 100% coexpressed NG2. Thus, we can establish that nestin-GFP expressing amygdaloid cells share the OPCs feathers by expression of NG2, outwardly rectifying currents, functional AMPA/Kainate subtype receptor, lacks glutamate transporter and also receive the synaptic input.

In several non-neurogenic areas, neocortex (Dawson et al., 2003), spinal cord (Horner et al., 2000), amygdala (Wennstrom et al., 2004), the majority of proliferating cells in situ expressed NG2. However, the differentiation potential of the NG2 expressing cells in situ in non-neurogenic areas seems to be restricted to glial cell lineage. On the contrary, in vitro study, at least subpopulation of postnatal NG2-expressing cells can generate neurons, astrocytes and oligodendrocytes (Belachew et al., 2003). NG2-expressing cells in SVZ also present self-renew in vitro, transit-amplifier type C-like multipotent cells features and transplanted early postnatal NG2+ cells in the postnatal hippocampus generate hippocampal GABAergic interneurons (Aguirre et al., 2004). Very recently reports demonstrated that NG2+/EGFP+ progenitors migrate from the SVZ through the RMS into the OB and the region-specific differences between NG2+ progenitor cells in SVZ, OB, cortex and cerebellum. Transplantation of NG2 expression cells from SVZ displayed these cells were migratory and give rise to glia and neurons in the OB. In contrast, cortical, OB, or cerebellar NG2 expression cells showed a very limited migratory potential and generate to glia in the subcortical white matter and striatum(Aguirre and Gallo, 2004). Kempermann's lab also found that a large proportion of the NG2+/BrdU+ cells continued over several weeks which might either indicate restricted differentiation in amygdala or that NG2+/BrdU+ cells already constitute a differentiated, yet proliferating phenotype. The

increasing in adult-generated oligodendrocytes over time was paralleled by a more pronounced decrease of BrdU+ NG2+ cells in the amygdala. In adult amygdala (Wennstrom et al., 2004) and adult neocortex (Dawson et al., 2003), the decrease of NG2+BrdU+ cells are also corresponded by a slight increase of BrdU+ cells expressing a mature oligodendroglial marker. Taken together, these data are consistent with the notion that adult-born NG2+ cells generate to oligodendrocytes in situ in amygdala.

The newly described ´receptor´ astrocytes (Matthias et al., 2003) expresses AN2, the mouse homology of the rat NG2 proteoglycan. ´Receptor´ astrocytes also displays other features as nestin-GFP-expressing cell in amygdala, for instance, outwardly rectifying currents, functional AMPA/Kainate subtype receptor expression, and no expression of glutamate transporter current. Although this reflects the homogeneous population of NG2 expressing cell might exist, functional properties are still different.

In the present study, the broad antigenic profile of the NG2+ cells suggest that nestin-GFP-expressing cell in amygdala constitute a progenitor cell population in vivo. Assuming that we examined the same cell population as in the study by Gallo's lab, in which the authors demonstrated a wider differentiation potential of NG2+ cells in vitro (Belachew et al., 2003) and in vivo (Aguirre and Gallo, 2004), this would support the concept of locally restricted differentiation potential in the adult amygdala. It seems that we observed a conflicted results. On one hand, we indeed found a few nestin-GFP-expressing cell in amygdala exhibit electrophysiologically neuronal characteristics, on the other hand, we never observed any NeuN expression in the NG2 cells. One explanation is NeuN has a limited sensitivity for the detection of mature neurons, as the example for cerebellar neurons (Mullen et al., 1992), thus we might have missed occasional adult-generated amygdaloid neurons using NeuN as a marker for mature neurons. The second explanation is the few 'neuron-like' nestin-GFP-expressing cells might be belonging to nestin and Dcx co-expression cells. Although this subpopulation is immature, they can receive the GABAergic input, as well as during adult neurogenesis in hippocampus in our previously study. The final explanation is the few `neuron-like` nestin-GFP-expressing cells contribute to a distinct subpopulation NG2 cells in amygdala, which share the same feature with the cortical subpopulation NG2 cells, however, not the NG2 cells in white matter (Chittajallu et al., 2004). Alternatively, NG2 might be expressed by a broad population, in which functionally different cell types were included. The proposed functions such as the generation of oligodendrocytes and/or other cell types might be carried out by different cell

populations, which just share the expression of NG2. Although in adult primates, the new neurons are generated in the amygdala (Bernier et al., 2002), concerning the few `neuron-like` nestin-GFP-expressing cells, we need further detailed study before we make the final conclusion.

4.5. Nestin-GFP-expressing cell display astrocytes features from corpus callosum to CA1

Except for the two physiologic neurogenesis regions: (DG) of hippocampus and the olfactory bulb, adult neurogenesis is also happened in otherwise non-neurogenic regions following ischemia (Arvidsson et al., 2002; Nakatomi et al., 2002). Administration of FGF-2 and EGF (Nakatomi et al., 2002),can induce endogenous neuronal progenitors from the posterior perventricle migrate to CA1 and regenerate neurogenesis. But whether or not there is the migration route exist from SVZ to CA1 under physiologic condition is still unknown.

Unpublished results from Kempermann's lab (data not shown) revealed that all nestin-GFP-expressing spongiform cells in CA1, characterized by protoplasmic astrocytes feature, displayed GFAP-immunoreactivity, S100β co-staining, none expressed early neuronal marker DCX (nor mature neuronal marker NeuN). In electron microscopy, these cells were characterized by a polymorphic nucleus with dispersed chromatin and strongly labeled thin processes branching out from the perinuclear region into different directions. Apart from free ribosomes many relatively large mitochondria were present in the cytoplasm. Also, bundles of intermediate filaments were observed in these cells characterizing them as protoplasmic astrocytes. Whole cell patch clamping study, we also found that nestin-GFP expressing spongiform cells displayed a current pattern characteristic for hippocampal astrocytes.

Another unique type of nestin-GFP-expressing cell, which was located in the corpus callosum/alveus, can be distinguished by a bipolar morphology with two long processes on opposite sides of the cell body. These bipolar cells consistently lacked GFAP-immunoreactivity. In the alveus/ corpus callosum, we also found that smaller nestin-GFP-expressing cell, which were characterized by their longish morphology and by the absence of elongated processes. 8% displayed co-localization with DCX, 29.3% of nestin-GFP-positive cells in the alveus were Ki67-immunoreactive, 42% of nestin-GFP expressing cells in the alveus shown nestin-GFAP co-staining. 27% of cells are nestin-GFAP-S100β

triple labeling. Both of the bipolar cells and the smaller cells shown the passive astrocytes feature by patch clamping study, which was characterized by their linear current voltage curve.

Two weeks after administration of the fluorescent dye 1,19-dioctadecyl-6, 69-di (4-sulfophenyl)-3,3,39,39-tetramethylindocarbocyanine (DiI) into the lateral ventricle, we also found DiI-positive nestin-GFP-expressing protoplasmic astrocytes in CA. Induced by environmental stimulation over 4 weeks, significantly increased the number of nestin-GFP-expressing protoplasmic astrocytes in CA1. The additional cells had not been born in CA1 was supported by the evidence that lacks BrdU-positive nestin-GFP-expressing astrocytes at 2 hours after BrdU injection and co-expressed cell-cycle associated protein Ki67 in nestin-GFP transgenic animals. Taken together all the results that we concluded that another migratory pathway may exist, which from SVZ to corpus callosum to CA1, where the multipotent precursor cells generate new protoplasmic astrocytes in an activity-dependent manner.

Although there is increased neurogenesis are found outside of SVZ and SGZ in certain pathological conditions, like ischemia, at least with administration of FGF-2, EGF, BDNF (Pencea et al., 2001a; Nakatomi et al., 2002; Chmielnicki et al., 2004), lacks evidences of physiologic neurogenesis in adult CA1 or striatum. We have discovered here that there is another migration route for multipotent precursor cells exist from SVZ to CA1, where they generate new protoplasmic astrocytes in an activity-dependent manner.

Compare with another neurogenic system from SVZ-RMS-OB, both of the routes origin from a (sub-) ventricular cell which with astrocytic properties. In the SVZ-corpus callosum-CA1 system, it is primarily astrocyte-like precursors that migrate in the corpus callosum/ alveus, and then migrate into CA1 generated to protoplasmic astrocytes, which is regulated by the environmental stimulation, as well as the regulation in the same experimental condition for adult neurogenesis in the dentate gyrus(Kempermann et al., 1997b). However, in SVZ-RMS-OB, it is a neuroblast that takes up migration and the adult olfactory neurogenesis not be regulated by the environmental stimulation (Brown et al., 2003b).

Although, neurogenesis and astrogenesis in the dentate gyrus and astrogenesis in CA1 originate from separate populations of precursor cells, both of the progenitor cells share a number of common features, such as co-expression of nestin and GFAP and with electrophysiological characteristics of astrocytes.

The absence of BrdU in nestin-GFP-expressing cells at early time points after i.p. administration (e.g. 2 hours) and co-expressed cell cycle associated protein Ki67 indicates that nestin-GFP spongiform cells are post-mitotic. Astrocytes play an important role in the modulation of neuronal excitability and synaptic transmission (Araque et al., 1998; Haydon, 2001). Our data suggest that there is a normal contribution of multipotent SVZ precursors to cellular plasticity in CA1. It seems that the migrating cells retain their multipotency for most part of their migration and also raising the possibility that if the local microenvironment was manipulated accordingly, they could be recruited into a neuronal fate. The interesting observation here is that under physiologic conditions this cellular plasticity in CA1 does not include neurogenesis, but only astrogenesis. Thus, in CA1 activity-dependent plasticity on the level of astrocytes makes sense, even though the details of the interaction with the plastic neurons of the same region are far from understood.

4.6. Nestin-GFP-expressing cells acquire the ´complex´ physiological phenotype after stroke and express AMPA/kainate glutamate receptors currents

Many evidences have demonstrated that nestin was marked upregulated expression in the damaged brain, including focal cerebral ischemia in adult rat (Lin et al., 1995; Duggal et al., 1997; Li and Chopp, 1999; Bond et al., 2002). Li and Chopp established that upregulated nestin expression persists for many weeks following brain ischemia and nestin immunoreactivity was present in large numbers of astrocytes and co-labeled with GFAP, particularly in the ischemic border zone (Li and Chopp, 1999). Lin et al. reported that strong and sustained expression of nestin was noted in GFAP-positive cells surrounding the lesion site following ischaemic and mechanical lesioning, therefore concluded that nestin expression was upregulated in reactive astrocytes (Lin et al., 1995). A recently reports shown that following spinal cord, nestin expression in a population of astrocytes of both the gray and white matters (Lang et al., 2004). Even though nestin was also expressed in neurons and oligodendrocytes after brain ischemia (Li and Chopp, 1999), nestin was considered as a marker for reactive astrocytes.

Reactive astrocytes are thought to play a major role in the healing process and potentially recapitulate ontogeny following brain injury, i.e. the re-expression of developmental proteins may indicate active reconditioning that could promote cell survival (Cramer and Chopp, 2000). In SVZ and SGZ, GFAP expression astrocytes have been identified as neural precursor cells (Doetsch et al., 1999b; Seri et al., 2001), this may indicate that, at least, subpopulations of astrocytes possess the ability to return to a more undifferentiated developmental stage in response to injury, in this point, the results from Lang et al. have indicated that the astrocytes in injured adult rat spinal cord may acquire the potential of neural stem cells (Lang et al., 2004).

Findings over the past decade have demonstrated the persistent neurogenesis in the adult brain, and adult neurogenesis can be regulated by environmental stimuli and also responsive to brain injury raised expecting for novel approaches to brain repair (Lowenstein and Parent, 1999; Peterson, 2002; Hallbergson et al., 2003; Picard-Riera et al., 2004). After ischemic tissue damage, neurogenesis is also happened in otherwise non-neurogenic regions (Arvidsson et al., 2002; Nakatomi et al., 2002).

Here, usage of transgenic mice that express GFP under control of the nestin promotor, we characterized the fate of nestin-expressing cells up to 8 weeks following 30 min occlusion of the middle cerebral artery (MCAo) and reperfusion by immunohistochemical and electrophysiological approach.

4.6.1. Nestin-GFP-expressing cells were upregulated following 30 min MCAo/ reperfusion, but Nestin-GFP-expressing cells and GFAP+ cells are distinct cellular populations

Both nestin and GFAP are generally regarded as markers of reactive astrocytes that are upregulated following brain injury. Lin et al. has demonstrated that GFAP+ reactive astrocytes co-expressed nestin and may play a major functional role in tissue healing process (Lin et al., 1995). In the present study, we found that nestin-GFP+ cells became upregulated in the ischemic lesion rim and core within 4 days (Fig.4.3) and the cells expressed the chondroitin sulfate proteoglycan NG2 (Fig.4.5, B) and nestin protein (Fig.4.4, B-E), but typically lacked astrocytic markers, ie. GFAP or S100β. Conversely, the majority of GFAP+ cells lacked nestin-immunoreactivity and surrounded the ischemic lesion. The majority of nestin negative and GFAP expressing cells are part of distinct cell populations in the damaged stratum following mild brain ischemia. At early time points after MCAo/

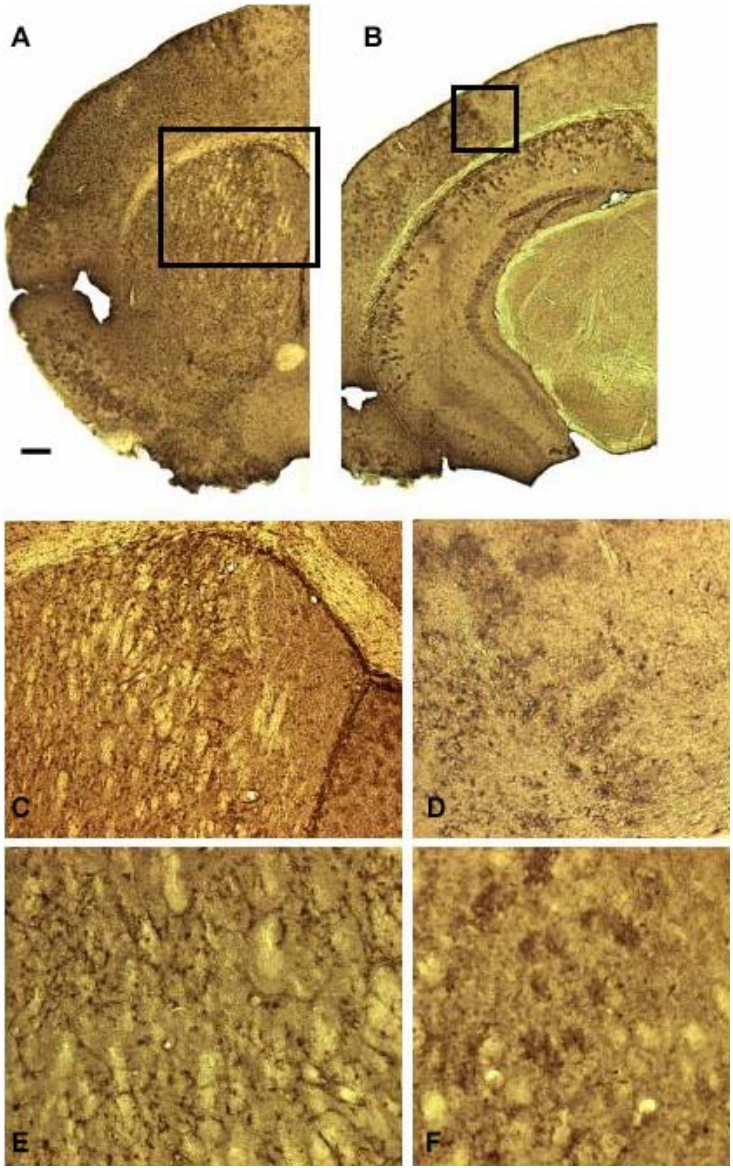


Fig. 4. 3. Nestin-GFP-immunoreactivity following cerebral ischemia.

Nestin-GFP mice were subjected to 30 min left MCAo and 4 days reperfusion. Nestin+ cells were identified by anti-GFP-immunostaining and visualization with DAB on coronal brain sections (40µm). Several pictures at 2.5 x objective have been joined together to yield composite images in **A** (ischemic hemisphere at bregma +0.9 mm) and **B** (ischemic hemisphere at bregma - 2.7 mm). **C** and **D**. Higher magnification of boxed areas in images above. Note the transition between intact tissue supplied by anterior cerebral artery and damaged tissue (middle cerebral artery) below corpus callosum (**C**) and in the cerebral cortex (**D**). **E** and **F**. Nestin-GFP+ cells in the in the ischemic striatum (**E**) and in the corresponding area in contralateral hemisphere **(F)**. Scale bar 300µm in A and B, 100 µm in C, 70 µm in D, 30 µm in E and F.

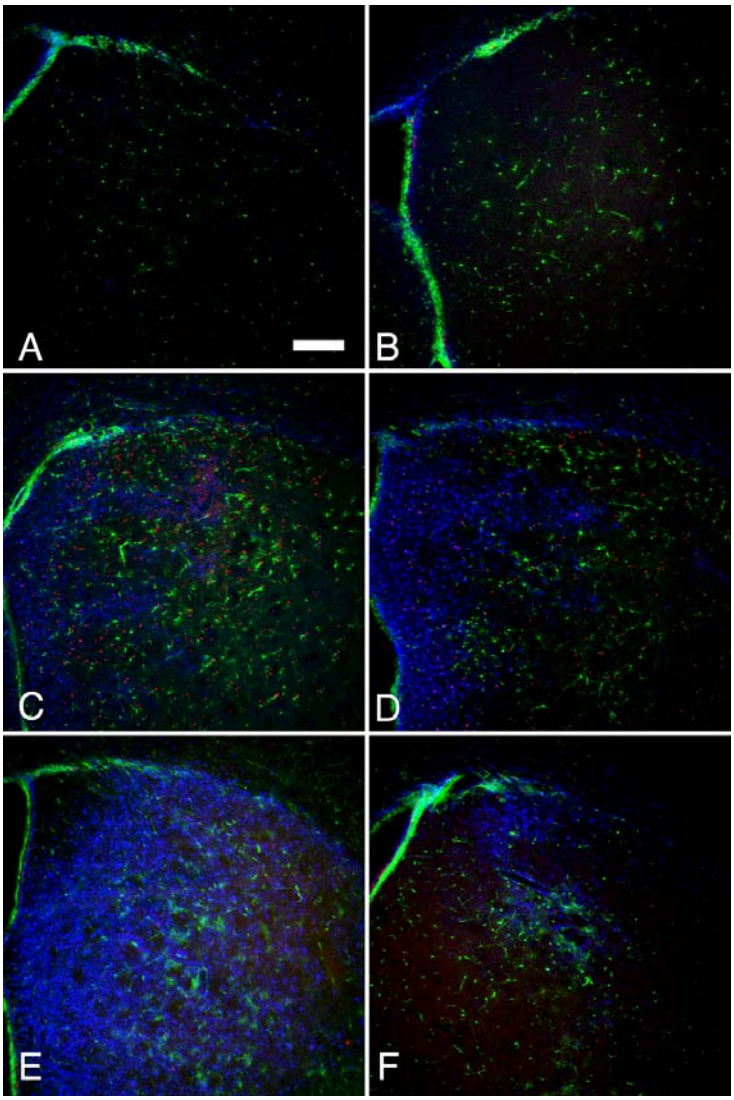


Fig. 4.4. Temporal and spatial distribution of nestin-GFP+, GFAP+ and Ki67+ cells following cerebral ischemia.

Nestin-GFP mice were subjected to 30 min left MCAo/reperfusion (B-F) or sham operation (A) and were sacrificed 1 (B), 2 (C), 4 (D), 7 (E), or 56 (F) days later. Triple-immunostaining (nestin-GFP+ cells: green; cell cycle associated protein Ki67: red; GFAP: blue) was performed on 40 µm coronal brain sections. A. Four days after sham operation, nestin-GFP+ and GFAP+ cells are concentrated primarily in the subventricular zone. Low numbers of nestin-GFP+ cells are located in the striatum (similar results in control brains, not shown). B. One day after MCAo/reperfusion, nestin-GFP- and GFAP-immunoreactivity increase. Nestin-GFP+ cells exhibit a plump morphology. Ki67+ cells are restricted to the subventricular zone and the corpus callosum/ alveus. C, D, E. Increased cell proliferation and reactive astrocytosis at 48 hours (C), 4 days (D) and 7 days (E) after MCAo/ reperfusion. Most GFAP+ cells lack nestin-GFP-immunoreactivity and *vice versa*. **F.** Glial scar at 8 weeks post MCAo/ reperfusion composed of nestin-GFP+ and GFAP+ cells. Scale bar 210 µm.

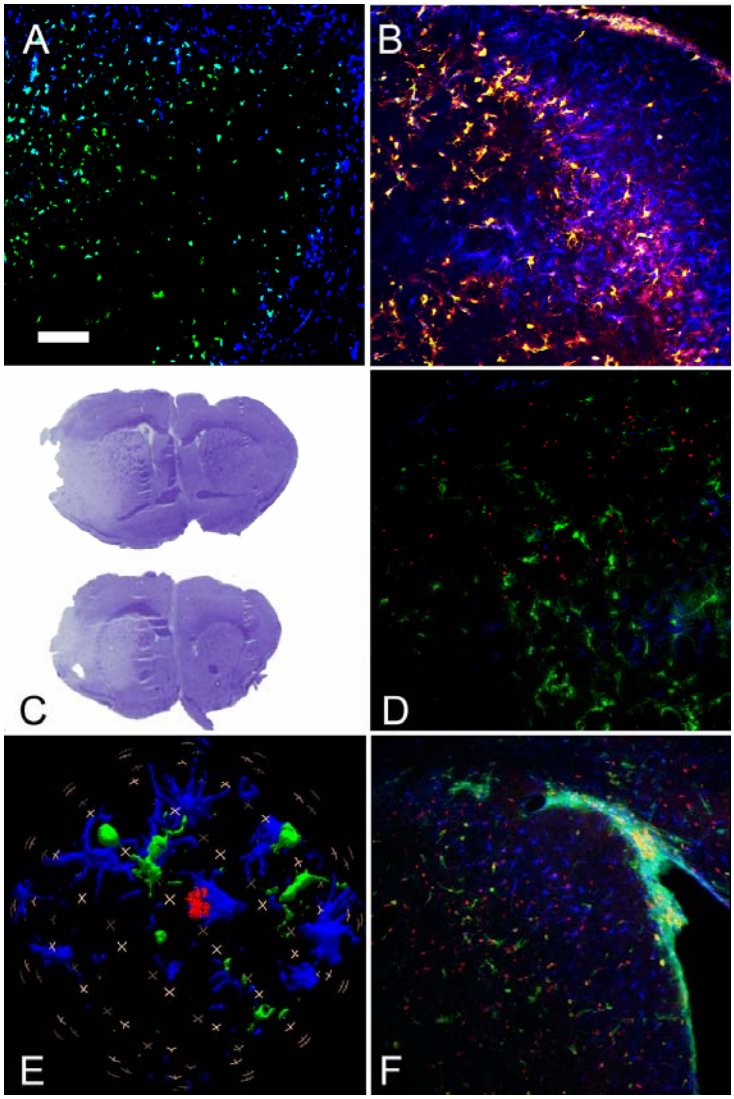


Fig.4.5. Different spatial distribution of nestin-GFP+ and of GFAP+ cells at early time points after stroke.

A. Nestin protein (green) and GFAP staining (blue) in 129/Sv wild-type mice 4 days after 30 min MCAo/ reperfusion. Note that nestin+ cells are located primarily within the striatal lesion and in a small transition zone between the ischemic lesion and the surrounding tissue while GFAP is typically found surrounding the lesion. Nestin-GFAP double-labeling is found predominantly in the lesion perimeter. The distribution of nestin- and GFAP-expression in this mouse strain thus closely resembles the pattern we found in GFP+ cells in our nestin-GFP transgenic animals, which have been back-crossed onto a C57/BL6 background (Fig.4.4, D). B. Virtually identical pattern of NG2 expression (red) and nestin-GFP-expression (green) at 4 days after MCAo/ reperfusion. GFAP: blue. C. Hematoxylin staining reveals typical morphology of lesions induced by 30 min MCAo/ reperfusion in nestin-GFP-transgenic mice. Note that medial striatum (ACA territory) has remained intact. D. TUNEL staining (red) at 4 days after MCAo/ reperfusion. Nestin-GFP+ cells (green) are located in close proximity to TUNEL+ nuclei. Note that GFAP+ cells (blue) are situated further away from area of intense TUNEL staining. E. Three-dimensional reconstruction of 30µm image stack of GFAP+ (blue)

cell lacking nestin-GFP (green) and co-expressing cell cycle associated protein Ki67 (red). F. Proliferating cells were labeled cumulatively over a period of 4 days after MCAo/ reperfusion with BrdU minipumps. The image shows that a fraction of GFAP+ astrocytes surrounding the lesion (blue) as well as the majority of nestin-GFP+ cells within the lesion (green) have incorporated BrdU (red). Scale bar 400 μm in A, 210 μm in B and F, 105 μm in D.

reperfusion only about 15% of nestin-GFP-expressing cells were co-labeled with GFAP, and vice versa only approximately 5% of GFAP-expressing cells were also nestin-GFP-immunoreactive. Nestin-GFP+ cells were located primarily within the ischemic lesion whereas GFAP+ cells were located in the surrounding tissue that had remained intact. We found high level of S100β co-expression (a marker for mature astrocytes) in GFAP+ cells around the lesion, but S100β immunoreactivity was virtually absent from nestin-GFP+ cells. In fact, GFAP/nestin-GFP-double positive cells were observed primarily in a small transitional zone.

4.6.2. Nestin-GFP-expressing cells proliferate in situ

Nestin-GFP+ cells, in the ischemic lesion, did not undergo apparent cell death by lacking expression of TUNEL up to 8 weeks in the lesion scar, although, nestin-GFP+ cells were intermingled within abundant TUNEL+ cells within the ischemic lesion (Fig. 4. 5,D). Compared to sham, the number of nestin-GFP+ cells ~ doubled after 7 days following MCAo, we found the nestin-GFP+ cells proliferated in the ischemic striatum, by the proof that the nestin-GFP+ cells shown the immunoreactivity against Ki67, a nuclear protein specifically expressed in proliferating cells(Gerdes et al., 1984) (Fig. 4. 5,E). After labeled all newly generated cells with BrdU via continuous administration by mini-osmotic pumps (Katchanov et al., 2001; Farah, 2004) in the animals 4 days after MCAo/ reperfusion. We also found that 84 ± 8 % nestin-GFP+ cells within the lesion were BrdU+ (n=4 animals; analysis of n=100 cells per animal) (Fig. 4. 5, F). This data imply that the vast majority of nestin-GFP+ cells had become BrdU+ after the ischemic insult. Considering the fact that by 4 days the number of nestin-GFP cells had almost doubled and that both the mother and daughter cell become BrdU+ after cell division we infer that on average each nestin-GFP+ cell must have undergone approximately one round of cell division after the ischemic insult, but they were not recruited in large numbers from the subventricular zone as indicated by absence of (i) co-labeling with intracerebroventricularly injected dye DiI and (ii) of migration chains (Data not shown). By contrast, BrdU colabeling was only found in

22 ± 3% of GFAP+ cells (n=3 animals, analysis of n=100 cells per animal), in which demonstrated that GFAP-expressing reactive astrocytes showed considerably less BrdU-incorporation as compared to nestin-GFP+ cells and majority of these cells may simply upregulated GFAP without a preceding cell division.

4.6.3. Nestin-GFP+ population acquires a uniform physiological phenotype after lesion

Based on their expression of membrane currents, electrophysiologically, nestin-GFP+ cells can be classified as displaying either “complex“ or “passive“ membrane properties and in control animals approximately half of the cells belonged to either population. The “complex” current pattern observed in nestin-GFP+ cells in the striatum is characteristic for also described for glial progenitor cells (Sontheimer et al., 1989), a newly described subpopulation of astrocytes in the CA1 region of the hippocampus (Matthias et al., 2003) and “complex astrocytes”, a subtype of astrocyte first described in the hippocampus (Jabs et al., 1994), a subpopulation of stem cells in the dentate gyrus (Filippov et al., 2003). The passive current profile, which was found in the majority of quiescent nestin-GFP+ cells in the striatum of control or sham-operated animals, is characteristic for the majority of astrocytes in the hippocampus (Steinhauser et al., 1992; D'Ambrosio et al., 1998). In response to the ischemic insult, the population of nestin-GFP+ cells shifts toward the “complex” physiological phenotype until 4 days after the insult the entire population exhibits a “complex” physiological phenotype. The absence of TUNEL-reaction in nestin-GFP+ cells and the fact that 24 hours following MCAo/ reperfusion, nestin-GFP+ cells with passive membrane properties were still detectable suggest that passive nestin-GFP+ cells had not undergone apoptosis or selective necrosis (so that complex cells would have selectively survived over time). Rather, we infer from out data that in response to the ischemic injury nestin-GFP+ cells with passive membrane properties transform into complex cells.

The complex nestin-GFP+ cells expressed AMPA-type glutamate receptors, which triggered a complex response. The activation of the cationic current represents the activation of the intrinsic AMPA receptor/channel response; the blockade of the resting K^+ conductance has been reported for astrocytes, Bergmann glial cells and oligodendrocytes (Kettenmann and Steinhäuser, 2005). The late transient activation of a K^+ conductance has not been reported before and seems to be unique property of nestin-GFP+ cells. The

"complex" cells lacked glutamate transporter activity similar as the hippocampus "complex astrocytes"(Matthias et al., 2003). Since glutamate uptake is a classic function of astrocytes, the "complex" cells are functionally distinct from the classic astrocyte and may serve different functions, which are, however, not yet understood. Since in the hippocampus "complex astrocytes" are characterized by the expression of AMPA-type glutamate receptors and by the lack of glutamate transporters (Matthias et al., 2003), we also tested for the presence of AMPA receptors and transporter activity in complex nestin-GFP+ cells in the striatum after MCAo/reperfusion. Whereas application of glutamate transporter substrate D-aspartate did not elicit a current, AMPA receptor ligand kainate resulted in a complex response. In summary, complex nestin-GFP+ cells after stroke show the same electrophysiological properties as complex astrocytes. By 4 days after the ischemic insult, all nestin-GFP+ cells analyzed displayed complex membrane properties. The absence of TUNEL-reaction in nestin-GFP+ cells and the fact that 24 hours following MCAo/ reperfusion, nestin-GFP+ cells with passive membrane properties were still detectable in large numbers suggest that passive nestin-GFP+ cells had not undergone apoptosis or selective necrosis (so that complex cells would have selectively survived over time). Rather, we infer from out data that in response to the ischemic injury nestin-GFP+ cells with passive membrane properties "actively" change their electrophysiological profile and transform into complex cells. Similar complex electrophysiological features have recently been described using Tg (GFAP/EGFP) mice in a subpopulation of hippocampal astrocytes. It has been hypothesized that "complex astrocytes" these cells might represent an intermediate "astron" cell type that retains some glial properties but has already switched on neuronal genes (Matthias et al., 2003). Importantly, a subset of these so-called "weakly fluorescent" GFAP-EGFP+ cells with complex membrane properties have been reported to express NG2 (vide supra; (Matthias et al., 2003). Taken together, nestin-GFP+ cells appear as a highly versatile cellular population largely distinct from GFAP+ reactive astrocytes. It is tempting to speculate that the electrophysiological changes after ischemia might reflect early steps toward a neuronal differentiation of nestin-GFP+ cells.

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SELECTED PUBLICATIONS

1.Filippov V*, Kronenberg G*, Pivneva T, Reuter K, Steiner B, **Wang LP**, Yamaguchi M, Kettenmann H, Kempermann G (2003) Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. ***Mol Cell Neurosci 23:373-382.***

2.**Wang LP**, Kempermann G, Kettenmann H (2005) A subpopulation of precursor cells in the mouse dentate gyrus receives synaptic GABAergic input. ***Mol Cell Neurosci 29:181-189***.

3.Kronenberg G*, **Wang LP***, Synowitz M*, Gertz K, Katchanov J, Glass R, Harms C, Kempermann G, Kettenmann H, Endres M (2005) Nestin-expressing cells divide and adopt a complex electrophysiologic phenotype after transient brain ischemia. ***J Cereb Blood Flow Metab***

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4.Glass R *, Synowitz M *, Kronenberg G, Walzlein JH, Markovic DS, **Wang LP**, Gast D, Kiwit J, Kempermann G, Kettenmann H (2005) Glioblastoma-induced attraction of endogenous neural precursor cells is associated with improved survival. ***J Neurosci 25:2637-2646.***

5.Shukla V, Zimmermann H, **Wang LP**, Kettenmann H, Raab S, Hammer K, Sevigny J, Robson SC, Braun N (2005) Functional expression of the ecto-ATPase NTPDase2 and of nucleotide receptors by neuronal progenitor cells in the adult murine hippocampus. ***J Neurosci Res 80:600-610.***

6.Golo Kronenberg, **Li-Ping Wang**, Harish Babu, Michael Synowitz, Paloma Vicens Caldéron, Gudrun Lutsch, Katja Reuter, Daniela Gast, Rainer Glass, Masahiro Yamaguchi, Helmut Kettenmann, Gerd Kempermann. Multipotent progenitor cells from the subventricular zone generate new astrocytes but no neurons in CA1 in an activity-dependent way ***(Submitted)***

7.Mami Noda, Yukihiro Kariura, Ulrike Pannasch, **Li-Ping Wang**, Yuki Kosai, Ayumi Sueyoshi, Bing Wang, Kaori Nishikawa, Tomoko Okada, Shunsuke Aoki, Helmut Kettenmann, Keiji Wada. Functional role of bradykinin; a new aspect of anti-inflammatory effects in microglia ***(Submitted)***

8.**Li-Ping Wang**, Golo Kronenberg, Karen Gertz, Andreas Philipps, Gerd Kempermann，Matthias Endres, Helmut Kettenmann. Mild MCAO results in a unique physiologic phenotype of striatal astrocytes. ***(Manuscript in preparation)***

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SELECTED SCIENTIFIC AND OTHER TRAININGS

1.Poster presentation, 03/2005, Gordon Research Conferences, Glial Biology:Functional Interactions Among Glia & Neurons. Ventura, CA, USA.

2.Poster presentation, 08/2004, Route 28 Summits in Neurobiology, Frauenchiemsee, Bavaria, Germany.

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8.Speaker, 01/2004, Graduate Program Colloquium 2004,Humboldt University Berlin, Germany.

9.Poster presentation, 11/2003, Society for Neuroscience 33rd Annual Meeting, New Orleans, USA.

10.Poster presentation, 09/2003, 6th European Meeting on Glial Cell Function in Health and Disease, Berlin, Germany.

11.Speaker, 04/2003,"Dynamics and Evolution of Cellular and Macromolecular Processes" Workshop Theoretical Biophysics 2003, Hiddensee, Germany.

EIDESSTATTLICHE ERKLÄRUNG

Ich versichere an Eides statt, dass ich die vorliegende Dissertation

Properties of Nestin-GFP-Expressing Cells in Different Regions of Adult Murine Brain

selbst und ohne unzulässige Hilfe Dritter verfasst habe, dass sie auch in Teilen keine Kopie anderer Arbeiten darstellt und die benutzten Hilfsmittel sowie die Literatur vollständig angegeben sind.

Berlin, den

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